THE CHEMISTRY OF THE CHROMOTROPIC ACID METHOD FOR THE ANALYSIS OF FORMALDEHYDE

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The Chemistry of the Chromotropic Acid Method for the Analysis of Formaldehyde

by

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ABSTRACT

Formaldehyde is present in the indoor air of many industrial and non-industrial environments. The procedure most commonly employed in North America for its analysis is one that uses chromotropic acid and concentrated sulphuric acid. The nature of the purple chromogen that is produced in the analytical procedure has not been fully understood until now. Evidence will be presented to support the hypothesis that the chromogen has a mono-cationic dibenzoxanthylium structure and not a para,para-quinoidal one that is commonly cited.

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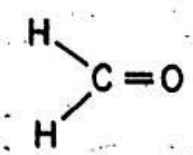
INTRODUCTION

Since its discovery in 1859 by Butlerov (1) formaldehyde [1] has become one of the most important and widely used industrial chemicals. In Canada alone in 1980 approximately 109,000 tons were produced (2). In 1983 approximately 830,000 tons were produced (3) in the U.S.. Formaldehyde is a colourless, flammable gas with a characteristic pungent odour. It is extremely irritating to the mucous membrane of the eyes, nose and upper respiratory tract even when present in concentrations as low as 20 parts per million (4). It has a boiling point of -19°C and a freezing point of -118°C (5). It is very soluble in water and combines readily with many chemicals. Anhydrous gaseous formaldehyde is not commercially available because it polymerizes easily and as a result, most formaldehyde is sold in the form of aqueous solutions ("formalin") containing 30-56% formaldehyde with 0.5-15% methanol as an inhibitor (6). It is also commercially available as the cyclic trimer trioxane [2], and as its linear low-molecular-mass homopolymer, paraformaldehyde [3].



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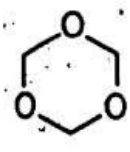

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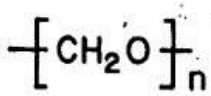


[3]

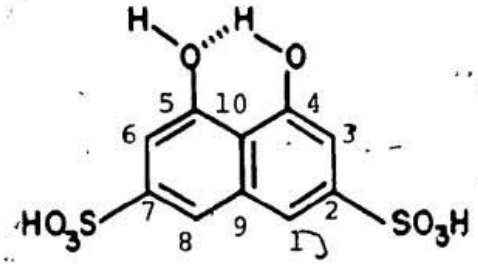
Commercially formaldehyde is produced by the air oxidation of methanol in the presence of a catalyst (1) e.g. silver or iron-molybdenum oxide. Formaldehyde is used primarily in the production of plastics and resins. In 1980 in Canada over 60% of the formaldehyde produced was used in the production (2) of urea-formaldehyde and phenol-formaldehyde resins. The former is used in the production of among other items, particle-board glues, hardwood plywood, furniture glues, textile finishes, fertilizers, thermal insulation, baking enamels, and wet-resistant papers. Phenol-formaldehyde resin is used in the manufacture of among other items, softwood and plywood glues, decorative laminates, thermal insulation (in fibreglass insulation), brake linings, varnishes and electrical components. The non-resin forms of formaldehyde are used to produce in addition to the above items, inks, explosives, pharmaceuticals, disinfectants, germicides, embalming fluids and preservatives (2).

The analysis of formaldehyde has long been of interest to everyone directly involved in the industry and to the occupational safety monitoring and regulatory

organizations. Approximately 60 occupational groups have been identified (8) as being potentially exposed to formaldehyde. In 1979 it was estimated (9) that in the United States approximately 1.40-1.75 million workers were occupationally exposed to formaldehyde. The data on the toxicity of formaldehyde is quite extensive (7,10,11). Although formaldehyde was long-suspected of being a potential carcinogen, it was only in 1980 that formaldehyde was linked to cancer in a study (12) in which rats were exposed to relatively high concentrations of formaldehyde.

The methods of analyses of formaldehyde depend largely on its amounts and its occurrence. In recent years the presence of significant amounts of formaldehyde in domestic indoor air has been recognized. This has been a result of formaldehyde being released from plywood, particleboard (13) and urea-formaldehyde foam insulation (U.F.F.I.) which had been extensively used (14) in retrofitting homes. Formaldehyde in air can be analysed by several different chemically-based procedures. Generally, these procedures first employ a calibrated pump to actively sample a known amount of the air to be tested into a

suitable liquid sorbent as a trapping medium. This is usually followed by treatment of the resultant solution with a reagent that is highly specific for formaldehyde and which produces a chromogen that can be quantitatively measured by ultra-violet or visible spectroscopy. Comparison of the observed absorbance to a reference calibration curve allows for an determination of the concentration of the formaldehyde in solution and hence in the air. In North America the most widely used chromogen-forming reagent with formaldehyde is chromotropic acid. Chromotropic acid [4] (4,5-dihydroxy-2,7-napthalenedisulphonic acid) is used in the method recommended by the National Institute for Occupational Safety and Health (NIOSH), Method P&CAM 125 (15,16).



[4]

The NIOSH procedure is based upon the earlier work of others (17,18,19). The limits of detection of

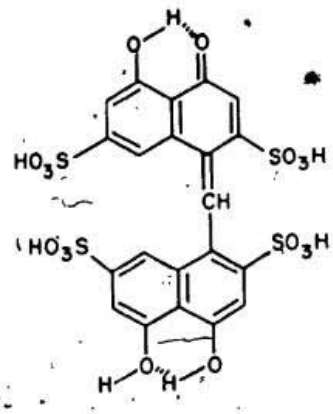
formaldehyde by the method using a simple uv-visible spectrophotometer are from 0.2 ug/mL to 10 ug/mL. For the analytical solutions used in the procedure these limits correspond to concentrations of formaldehyde of between 6.7×10^{-6} M and 3.3×10^{-4} M. A recent development in which the thermal lens effect (20) using a laser is employed permits a lower limit of detection of formaldehyde in the analytical solution of as low as 1.5×10^{-8} M, an approximately 400-fold enhancement of sensitivity.

There are other methods employing reagents that are more sensitive with ordinary spectrophotometers, and are subject to fewer known interferents than chromotropic acid. One that was widely used in Canada during the recent concern over formaldehyde release in U.F.F.I.-insulated homes employs pararosaniline (PRA) (21,22) as the reagent which forms a chromogen with formaldehyde. This method avoids the use of concentrated sulphuric acid which is essential in the chromotropic acid procedures. However, it was only very recently that it was shown that samples of formaldehyde collected in water as a trapping medium could be stabilized with small amounts of PRA (23,24) and hence

be analysed by the PRA method (21,22). Prior to that, dilute environmental formaldehyde solutions could only be stabilized by the addition of bisulphite (25), or bacteriocides such as mercury (II) chloride, tin (II) chloride or sodium pentachlorophenate (26) and as such could only be analysed using the chromotropic acid procedures. The recent findings could possibly lead to a lessening of the importance of the chromotropic acid method in the future.

The use of chromotropic acid as a formaldehyde-specific reagent was first described by Eegriwe in 1937 (27). It has known interferents as determined by Altshuller et al. (19) when used in gas analyses. These interferents however are usually not significant where formaldehyde is usually encountered. The nature of the chromogen and its formation by the reaction of chromotropic acid with formaldehyde has never been unambiguously proven. The most often-quoted structure (15,16,28,29,30) for the chromogen formed between chromotropic acid and formaldehyde has been the para,para-quinoidal adduct [5]. No direct proof of this structure has ever been presented. The structure is based

on an analogy with the reaction of formaldehyde with aromatic compounds in the LeRosen test (28).



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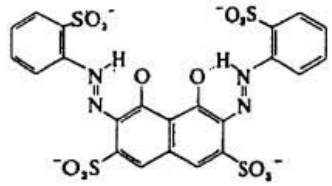
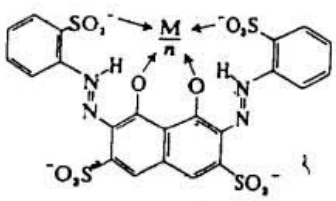


[6]

A major objection to this structure would appear to be the fact that chromotropic acid is known to form a large number of compounds with diazotised derivatives of aromatic compounds. In all of these cases, the structures presented (for which very little direct proof has been advanced) have diazo groups undergoing substitution in the position ortho to both the hydroxyl and sulphonic acid groups. An example is provided by Sulfonazo III [6] (31). The strongest evidence for this type of structure is that it is consistent with its chemical properties. It is a strong complexing agent for Ca^{2+}, and other metal ions

presumably via the formation of [7]. The alternate structure [8] would not involve the chelating potential of the phenolic hydroxyl groups (31).



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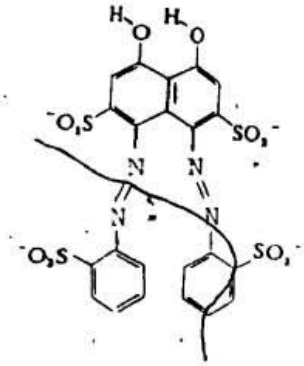
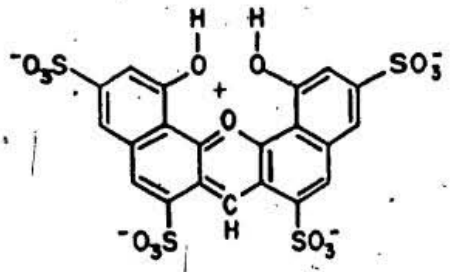


[8]

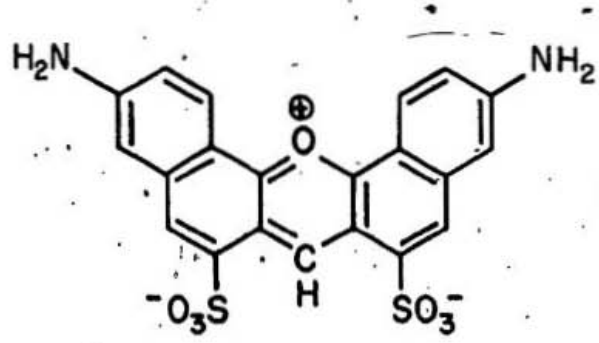
An alternative structure that has been proposed by Kamel and Wizinger (32) but which appears to have been ignored in the recent literature and for which there are only three brief citations (33,34,35) is [9]. This mono-cationic dibenzoxanthylium structure was proposed by analogy with the product obtained [10] when J-acid [11] (7-amino-4-hydroxy-2-naphthalenesulphonic acid) reacts with formaldehyde in concentrated sulphuric acid.



[9]

Carbon, hydrogen and sulphur analyses were consistent with the proposed structure [10]. No other direct evidence was presented for [10]. The analogous formaldehyde-chromotropic acid adduct [9] could not be isolated from the reaction mixture since it was much more water soluble than [10] and thus precluded a similar carbon, hydrogen and sulphur analysis on it.

The work described in this thesis was aimed at understanding the nature of the chemistry and establishing the structure of the chromotropic acid-formaldehyde chromogen. It was felt that a better understanding of the chemistry of the reaction between chromotropic acid and formaldehyde could lead to a modification of the method such that concentrated sulphuric acid could be eliminated from the method.



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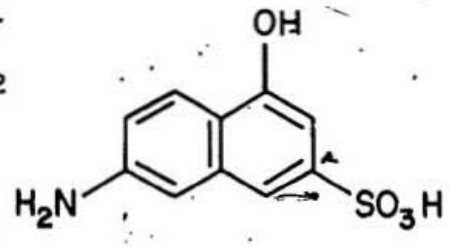


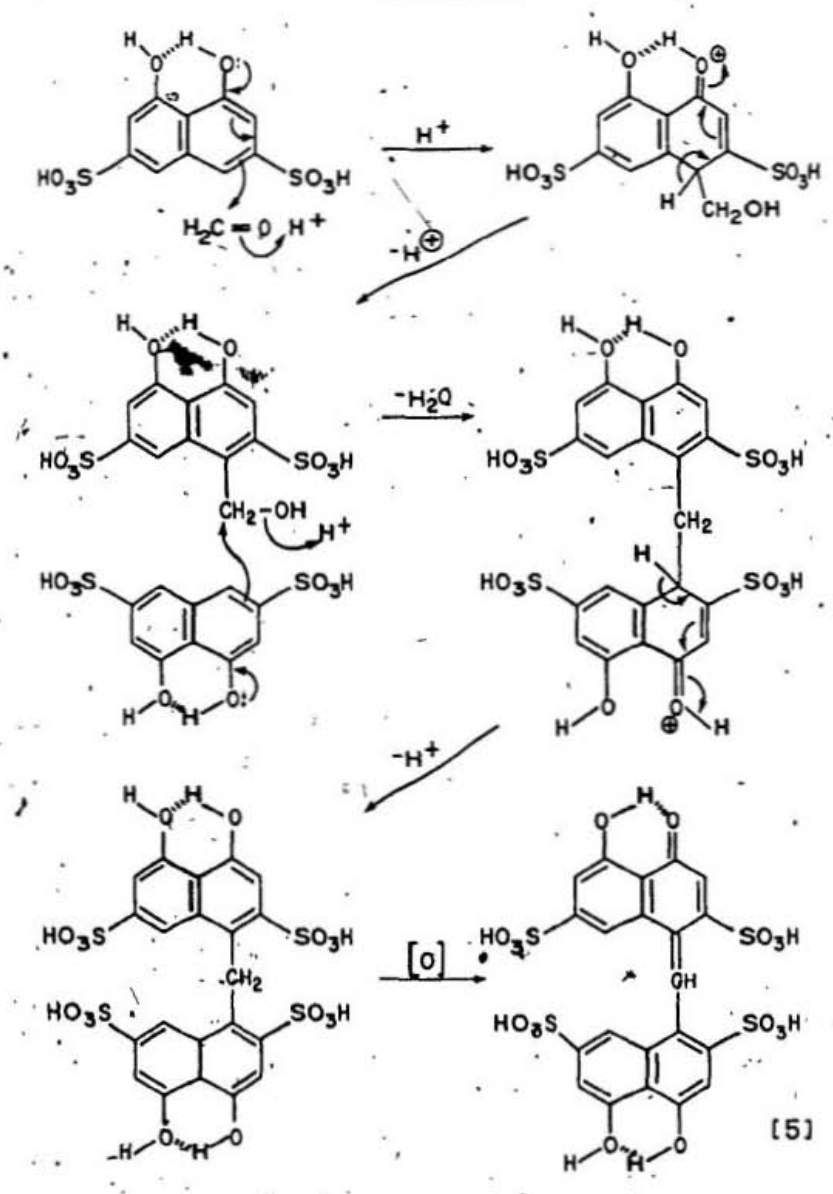
[11]

CHAPTER 1

In the NIOSH chromotropic acid procedure, concentrated sulphuric acid is used in the development of the chromogen. The following outlines the steps that are conducted in a recent modification (36) to the basic NIOSH procedure: Aqueous 5% chromotropic acid (300 uL) is added to a 2.0 mL-aliquot of the sample of formaldehyde in either distilled or deionized organic-free water solution, or in 1% aqueous bisulphite solution (used as a stabilizer). The mixture is thoroughly mixed and 3.0 mL of concentrated sulphuric acid is added. After thorough mixing the solution is heated in a boiling water bath for 1 h. The purple solution is allowed to cool to room temperature. An aliquot of the solution is removed and its absorbance is measured at 580 nm. The reference solution is a blank containing no formaldehyde but which is treated in the identical manner as those solutions which do. The resulting absorbance is compared with a calibration curve produced using five standard solutions, and the concentration of the formaldehyde is then calculated.

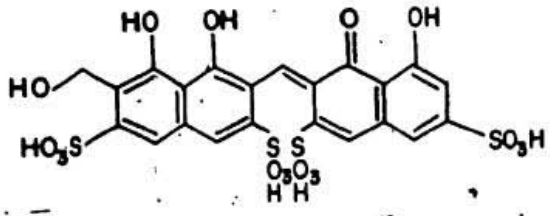
One of the structures proposed for the chromogen that is formed is the para,para-quinoidal adduct [5]. Its formation can be envisioned by the mechanism outlined in Scheme 1:

Scheme 1

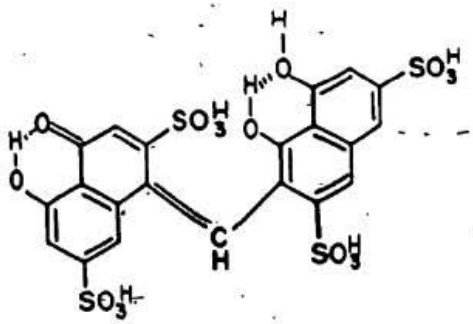


[5]

It can be seen that the alternative ortho, ortho-quinoidal adduct [12], and/or the ortho,para -quinoidal adduct [13] could in principle also be formed by a similar pathway.



[12]



[13]

In order to determine the exact position on the chromotropic acid molecule where condensation of formaldehyde occurs it was determined that extensive analysis using NMR spectroscopy would be required. The ^{1}H-NMR spectrum of chromotropic acid in deuterium oxide solution is shown in Figure 1.

Figure 1

The 60 MHz spectrum is a typical AX-type (37), with two sharp doublets at 7.64 and 7.24 ppm with J-2Hz. The assignment of these signals to the appropriate protons cannot be made unambiguously by only considering neighbouring group effects. Of the two types of protons on the chromotropic acid molecule one (on carbon C-3 or C-6) is ortho to both the hydroxyl and sulphonic acid group, and the other (on carbon C-1 or C-8) is para to the hydroxyl but ortho to the sulphonic acid group. Since these two types of protons are both ortho to the sulphonic acid group the effect of the electron-withdrawing sulphonic acid group is the same on both of them. The hydroxyl group on the other hand is ortho to the proton on C-3 (or C-6) and para to the proton on C-1 (or C-8). However, for phenol it has been shown that the ortho and para position protons are equally affected by the hydroxyl group and have similar chemical shifts (38). In naphthalene (38) itself the alpha-protons appear downfield (7.81 ppm) with respect to the beta-protons which appear at 7.46 ppm. A ring-current effect (38) can account for this assignment. By analogy with napthalene therefore, the chemical shift at 7.64 ppm can be assigned to the proton on C-1 (or C-8) and the,

chemical shift at 7.24 ppm can be assigned to the proton on C-3 (or C-6). Nevertheless, the assignment of chemical shifts using only the arguments made above is not sufficient for a multi-substituted napthalene derivative such as chromotropic acid.

It was observed during the ^{1}H-NMR experiments on chromotropic acid in deuterium oxide that with time the signals at 7.24 ppm gradually diminished in intensity. Ultimately, the signals disappeared completely and the doublet at 7.64 ppm was converted into a sharp singlet. This finding could be reproduced within 0.5 h when the NMR tube containing the chromotropic acid was heated in a boiling-water bath. The ^{1}H-NMR spectra from a typical experiment are shown in Figure 2.

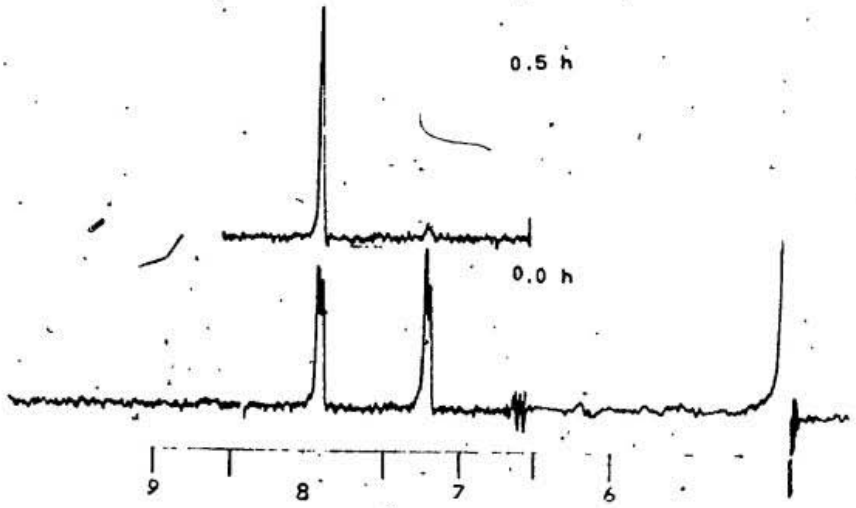
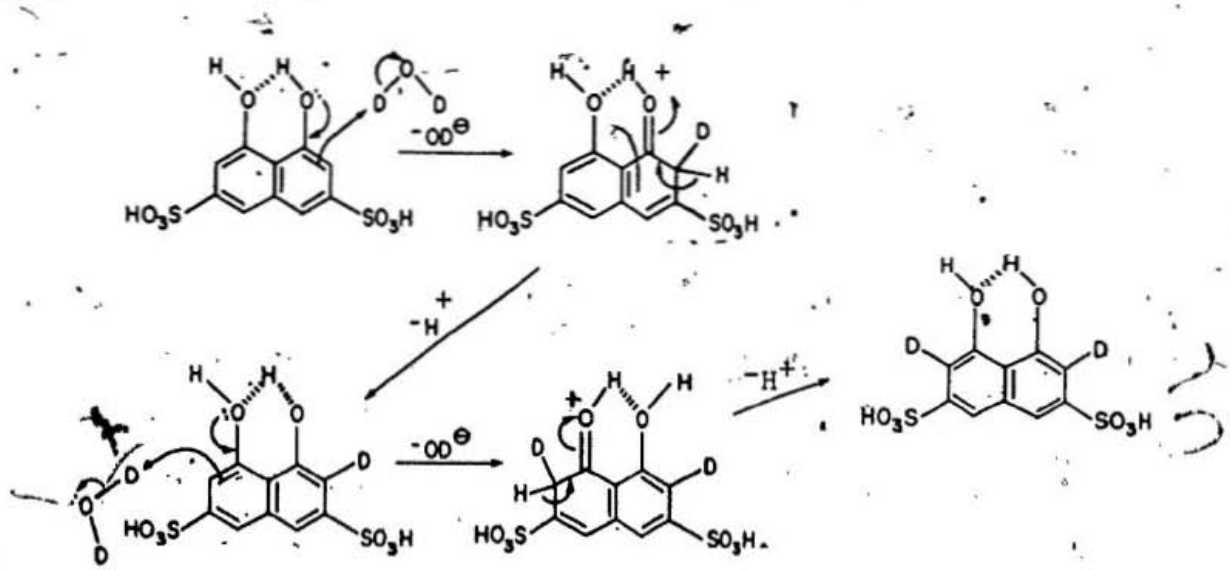


Figure 2

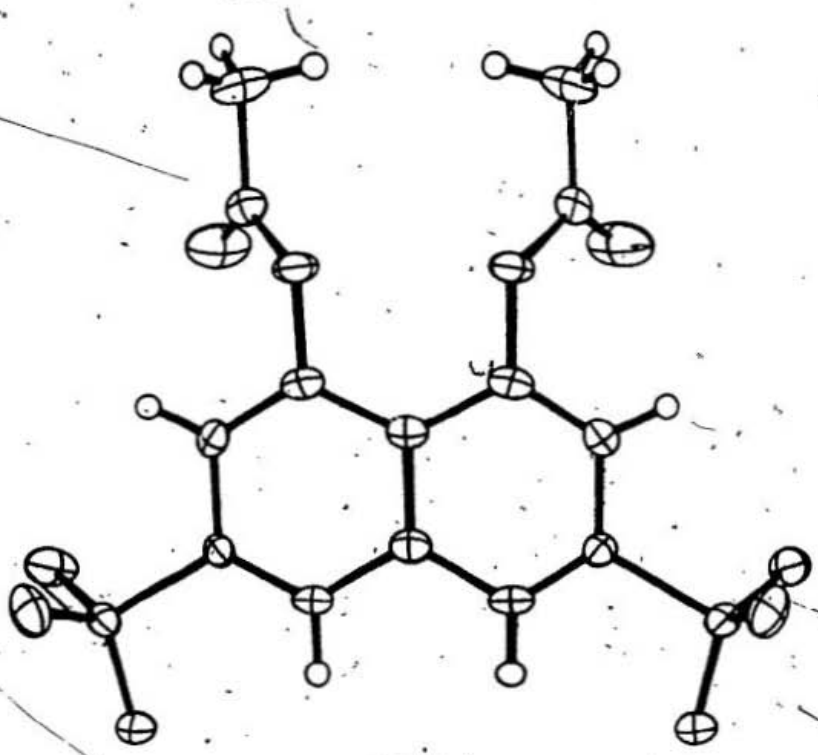
This finding could be accounted for by the pathway shown in Scheme 2. Experiments showed that only the signals at 7.24 ppm could be made to undergo exchange in deuterium oxide. That a simple hydrogen-deuterium exchange occurs was established by adding water to the solution contained in the NMR tube. In this way the original ^{1}H-NMR spectrum could be regenerated. Up to this point unequivocal assignment of the ^{1}H-NMR signals had not yet been achieved. Therefore, even though the proton in the ortho position to the hydroxyl group is indicated in Scheme 2 as being exchanged by a deuterium atom, the exchange of the para proton could be considered to be equally likely.

Scheme 2



The fact that only one position on the chromotropic acid molecule undergoes facile deuterium exchange suggested that only two possibilities for a quinoidal formaldehyde-chromotropic acid adduct, namely the para,para-quinoidal adduct [5], or the ortho,ortho-quinoidal adduct [12] could exist. In order to unequivocally assign the ^{1}H-NMR signals to their respective protons in chromotropic acid, it was decided to synthesize the corresponding diacetate of chromotropic acid. The proximity of the methyl protons of the acetoxy group to the proton on C-3 (or C-6) suggested that a nuclear Overhauser effect (NOE) (39) in the ^{1}H-NMR spectrum could be observed.

Since chromotropic acid is extremely water-soluble, its acetylation was carried out by reacting it in agueous sodium hydroxide with excess acetic anhydride (40). The reaction afforded a product that could be crystallized. Spectral data were consistent with it being the diacetate of the disodium salt of chromotropic acid [14]. The free acid form could not be crystallized. An X-Ray crystallographic analysis of [14] is shown in Figure 3.



[14]

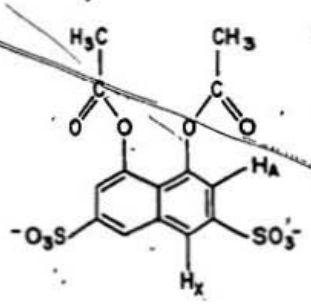


Figure 3

This analysis was conducted as it was hoped that the ultimate direct proof of structure of the chromogen, if it could be suitably crystallized, would come from its X-Ray crystallographic analysis.

The 60 MHz ^{1}H-NMR spectrum consists of a single signal at 2.19 ppm due to the acetate methyl groups and a pair of sharp AX-type doublets at 8.27 and 7.48 ppm. That these low-field signals were due to the C-1, C-8 protons and the C-3, C-6 protons respectively was established by a series of NOE difference experiments conducted with a 300 MHz NMR spectrometer. These are shown in Figures 4a-d.

Figure 4a is the 300 MHz ^{1}H spectrum of (14). The solvent used was D_2O. The signal at 4.8 ppm is due to HOD. In Figure 4b irradiation of the 8.27 ppm signal enhances the signal at 7.48 ppm relative to the acetate signals at 2.19 ppm. In Figure 4c irradiation of the signal at 7.48 ppm enhances the acetate signals relative to the signal at 8.27 ppm. In Figure 4d irradiation of the acetate signal clearly enhances the signal at 7.48 ppm relative to that at 8.27 ppm.

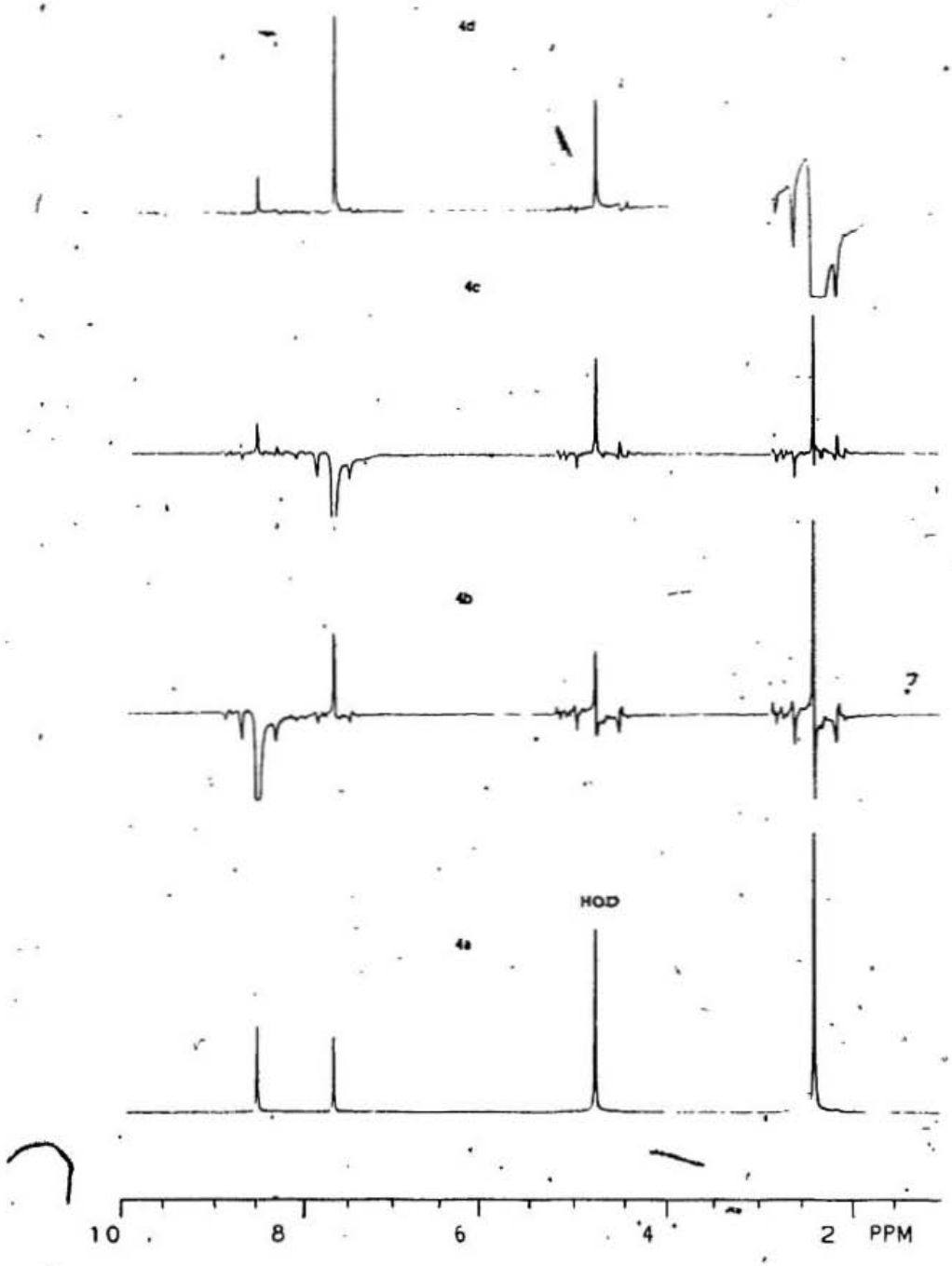


Figure 4

As a result of this NOE experiment the 8.27 ppm signal can be assigned to the proton at C-1 (and C-8) and the signal at 7.48 ppm to the proton at C-3 (and C-6). That is, the higher-field signal is due to the proton ortho to the acetoxy group. By analogy therefore with chromotropic acid itself the higher-field signal which undergoes deuterium exchange is that due to the proton ortho to the hydroxyl group.

The ^{13}C-NMR spectrum of chromotropic acid had been assigned previously by Lajunen et al. (41) The spectrum which is shown as Figure 5 consists of six signals.

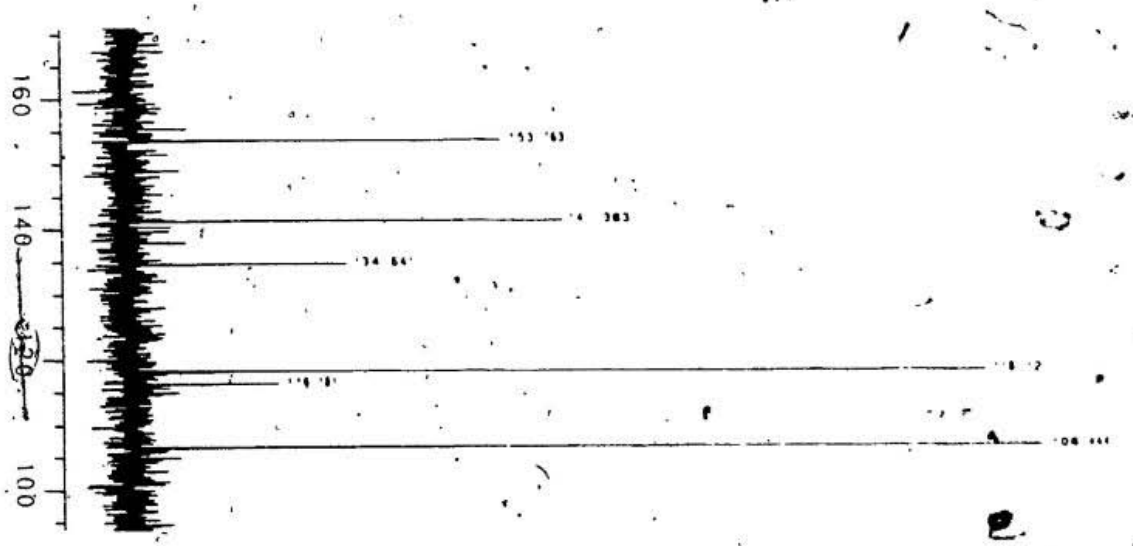


Figure 5

Lajunen et al. assigned the signals to the respective carbon atoms by studying the changes in the ^{13}C chemical shifts caused by changing the pH of the solutions. The largest effect on the chemical shift was observed for the C-4, C-5 and C-10 signals when the pH was changed from 4.5 to 8.0. At basic pH one of the hydroxyl groups is dissociated into the corresponding oxyanion. Dissociation of the second hydroxyl group is not possible in aqueous solution since it is very strongly hydrogen-bonded to the neighbouring oxyanion via an intramolecular six-membered ring. The assignment of all carbons except C-1 (and C-8) and C-3 (and C-6) were reasonably unambiguous. Since it was hoped that ^{13}C-NMR spectroscopy would assist in the structure determination of the chromogen formed between formaldehyde and chromotropic acid this ambiguity was not acceptable. Therefore a two-dimensional ^{1}H-^{13}C chemical shift correlation (39) experiment was undertaken on chromotropic acid. The experiment is shown as Figure 6.

As can be seen, the lower-field signal at 7.94 ppm due to the proton on C-1 (and C-8) in the 300 MHz ^{1}H-NMR spectrum correlates with the signal at 118.1 ppm in the 75 MHz ^{13}C-NMR spectrum. This is in agreement with the

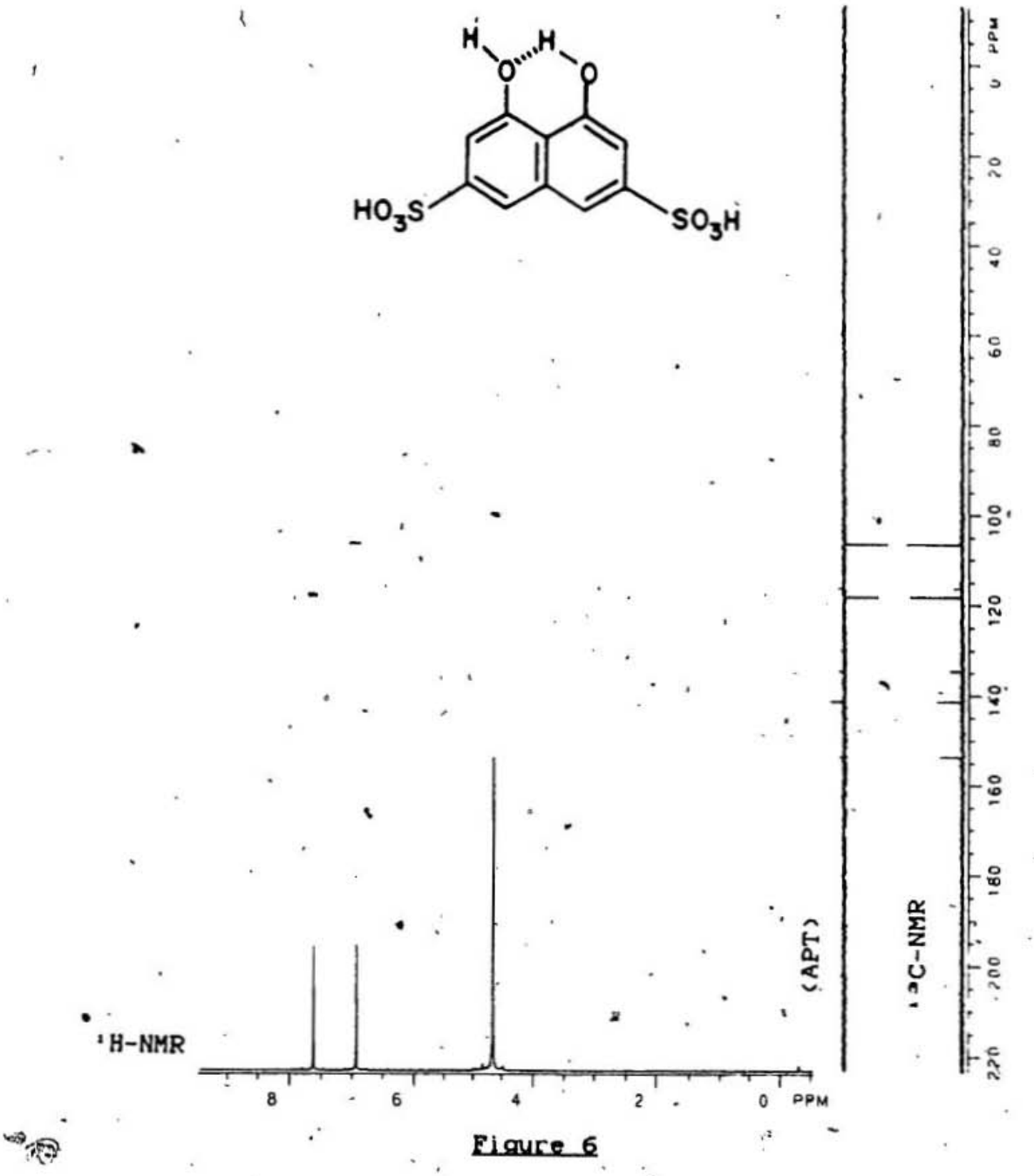

HO3S
SO3H
H
O
H
O
1H-NMR
8
6
4
2
0
PPM
(APT)
13C-NMR
0 PPM
20
40
60
80
100
120
140
160
180
200
220

Figure 6

assignment made by Lajunen et al. Also the ^{1}H signal at 7.24 ppm due to the proton on C-3 (and C-6) correlates with the ^{13}C signal at 106.4 ppm. In addition, the applied proton test (APT) (39) shown in Figure 6, and the ^{1}H-^{13}C spin-spin coupled spectrum are consistent with these assignments. Finally, the ^{13}C-NMR of the deuterium-exchanged chromotropic acid revealed that the intensity of the signal at 106.44 ppm was diminished by over 90%. All other signals were unchanged.

This spectroscopic evidence strongly suggested that it was the C-3 (and C-6) position of chromotropic acid that was the most reactive for the deuterium exchange noted earlier. Thus it would imply that it is the ortho,ortho-quinoidal adduct [12] and not the para,para-quinoidal adduct [5] that would be formed. The objective of this phase of the work was to unambiguously define the NMR spectrum of chromotropic acid in order to facilitate further positive identification of the chromogen. This objective was met.

CHAPTER 2

Having defined the ^{1}H-NMR spectrum of chromotropic acid it was felt that the most information as to its reaction with formaldehyde could be obtained by following the course of the reaction directly in a NMR spectrometer. The analytical solution contains a large excess (from approximately 240- to 3,000- fold molar excess) of chromotropic acid relative to formaldehyde. The sensitivity of an ordinary NMR spectrometer would not be great enough to detect the presence of the reaction product(s) in the presence of such a large excess of one of the reagents. Furthermore the low concentrations employed in the analytical procedure precludes the use of even a high-resolution Fourier-transform NMR instrument. This was confirmed in preliminary experiments in which both high-resolution Fourier-transform ^{1}H- and ^{13}C-NMR spectrometry of the analytical reactions were examined. Therefore experiments were conducted with concentrations of reagents which would permit direct observation in a 60 MHz NMR spectrometer. Molar ratios of formaldehyde to chromotropic acid of only 1:1 and 1:2 were employed.

The reactions at either molar ratio in the presence of catalytic amounts of sulphuric acid were found to be too rapid to monitor by NMR. Therefore it was decided to follow the reaction in the absence of sulphuric acid. The reaction solvent was water. A 1:2 molar ratio of formaldehyde to chromotropic acid (1.0 mL of an aqueous 1.3 M solution of formaldehyde, and 1.0 g of disodium salt of chromotropic acid in 15 mL of deionized organic-free water) was stirred at reflux temperature. A dark red colour formed at the onset of refluxing. Aliquots of the reaction mixture were removed periodically and were evaporated to dryness. The residues were redissolved in deuterium oxide and their 60 MHz NMR spectra were recorded. Preliminary experiments indicated that after overnight refluxing the protons in the aromatic region of the NMR spectra were no longer present. That the reaction appeared to have undergone polymerization was evident by the fact that the reaction mixture became very viscous. A typical series of spectra that were recorded is presented in Figures 7a-e.

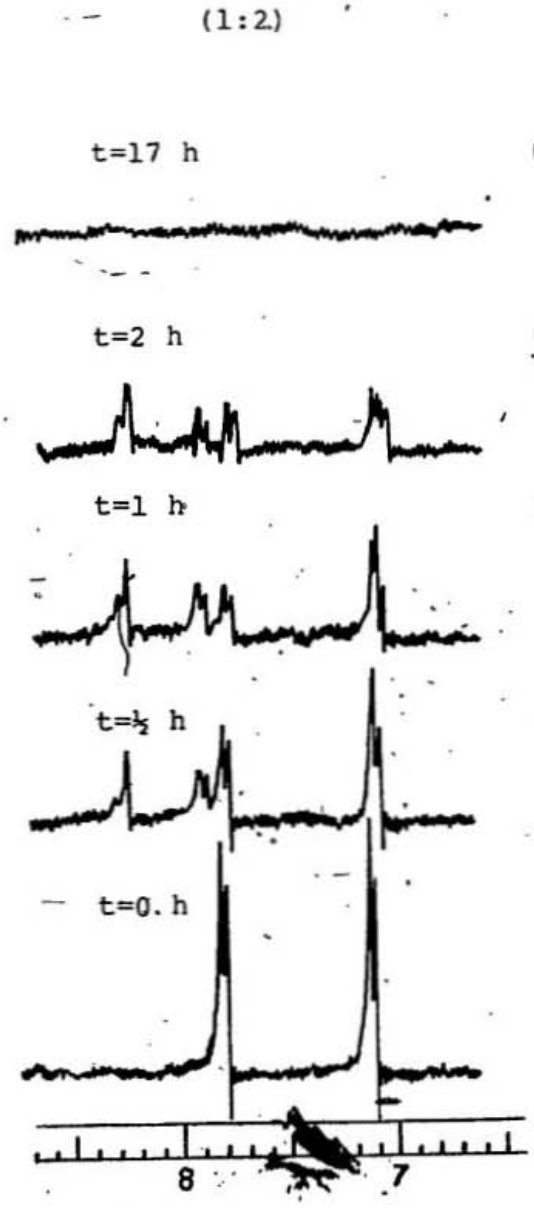


Figure 7

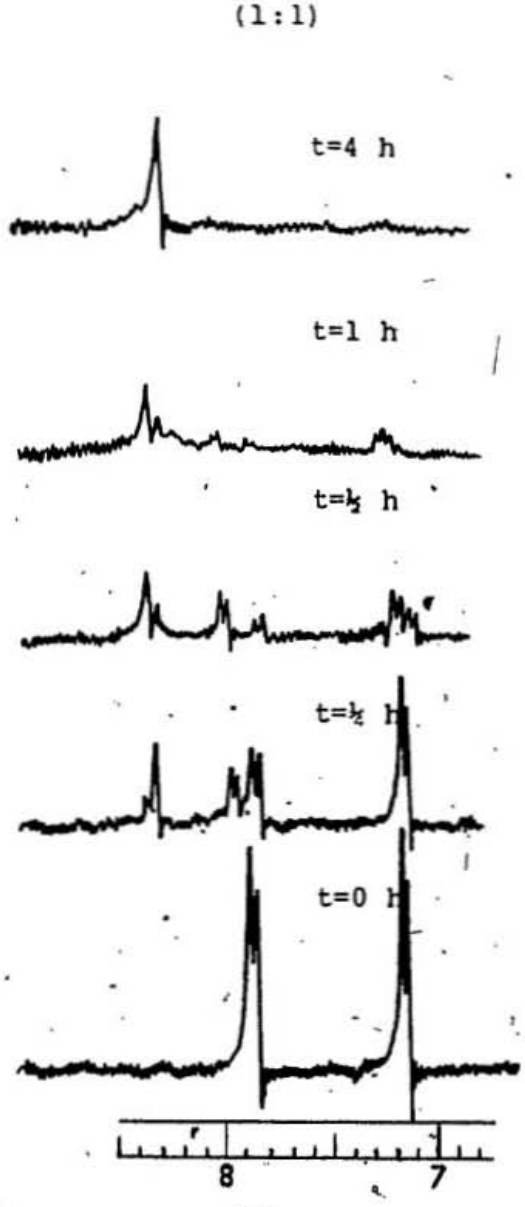


Figure 8

Figure 7a is the NMR spectrum of the reaction mixture at t=0 h. Apart from the chromotropic acid signals, a shoulder to the residual HOD or H_2O peak is barely perceptable, and is due to the hydrated form of formaldehyde. Figure 7b shows the spectrum which was recorded at t=0.5 h. In the aromatic region new signals at 8.53 and 8.10 ppm appear,, and there are changes in the chemical shifts and the line shapes of the chromotropic acid signals. In addition, a new signal appears at 5.43 ppm presumably due to methylene protons. Figure 7c shows the spectrum which was recorded at t=1.0 h. The lowest field signals are seen to consist of two singlets, a smaller one at approximately 8.60 ppm, and the larger one at 8.53 ppm. The signals centered at 8.10 ppm consist of two doublets each having similar coupling constants, J=2 Hz. The signals centered at 7.40 ppm consist of two overlapping doublets, of similar intensities and coupling constants, J=2 Hz. Figure 7d shows the spectrum which was recorded at t=2.0 h and which shows no major significant changes occuring. Similar spectra were obtained at t=3.0 and 4.0 h. The last spectrum presented as Figure 7e shows the spectrum recorded after overnight refluxing. All the aromatic region signals

apparently disappeared. The high viscosity of the solutions could account for this last observation.

The observations in general were initially confusing as they suggested that it was the C-1 (and C-8) position on chromotropic acid that was the primary site of reaction with the formaldehyde. That is, the intensity of the signals due to the proton(s) at C-1 (and C-8) was apparently diminishing relative to that of intensity of the signals due to the proton(s) on C-3 (and C-6). This would be a direct contradiction to the findings noted earlier with respect to the deuterium exchange experiments.

When a 1:1 molar ratio of formaldehyde to chromotropic acid (or when a large excess of formaldehyde relative to chromotropic acid was used) was reacted and followed as above, a different series of NMR spectra was obtained. A typical series is presented in Figures 8a-e. Figure 8a is the NMR spectrum of the reaction mixture at t=0 h and it is similar to Figure 7a. Figure 8b shows the spectrum which was recorded at t=0.25 h. This spectrum is similar to that observed at t=0.5 h in Figure 7c. Figure 8c shows the spectrum which was recorded at t=0.5 h. Figure 8d shows the spectrum recorded at t=1.0 h. This spectrum

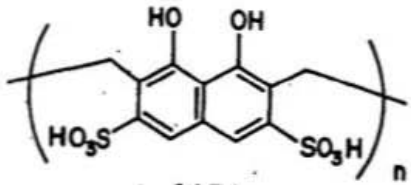
indicates that the chromotropic acid has nearly all been consumed as the signals centered at 7.50 ppm have all but disappeared. The lowest field signals consist of two singlets, as noted in Figure 7c, except that now the lower field singlet was the larger of the two. In addition a methylene peak centered at 5.63 ppm is evident. Figure 8e shows the spectrum recorded at t=4.0 h. The aromatic region signals have all disappeared except for a sharp singlet which is at 8.12 ppm. The signal due to the methylene protons is still present but is at 4.95 ppm.

These observations are similar to those made for the 1:2 molar ratio conditions described above, except that a cleaner reaction mixture was apparently formed when the 1:1 conditions were employed.

Attempts at separating the components of the reaction mixtures obtained under either sets of conditions proved to be futile. Thin-layer chromatography (TLC) on silica gel or cellulose (42) could not resolve the mixtures. Neither could paper chromatography (43), high performance liquid chromatography (HPLC) using simple, ion-pair reversed-phase chromatography, with (44) and without (45) the addition of ion-pairing counter ions. All

attempts to crystallize the product(s) were also unsuccessful.

The proton-decoupled ^{13}C-NMR spectrum of the crude product obtained from the 1:1 conditions was very simple with only 7 signals evident. Preliminary analyses of this spectrum suggested that the signal due to C-3 (and C-6) in chromotropic acid was the most affected with its chemical shift changing by 12.84 ppm, from 106.44 ppm in chromotropic acid to 119.28 ppm in the reaction product. The additional signal at 26.94 ppm is presumably due to a methylene carbon (46). The simplicity of the spectrum suggested that a highly symmetrical product of chromotropic acid was formed. In addition only a singlet in the aromatic region at 8.10 ppm and a singlet at 4.85 ppm in the 300 MHz ^{1}H-NMR spectrum was observed. This spectral evidence suggested that the crude product obtained is a linear polymer of formaldehyde and chromotropic acid linked in the ortho,ortho positions as shown in structure [15]. The two-dimensional ^{1}H-^{13}C chemical shift correlation spectrum of [15] is shown in Figure 9.


HO
OH
HO3S
SO3H
n

[15]

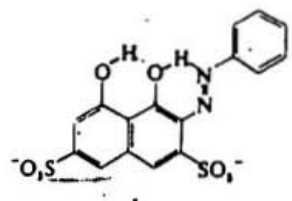
8
6
4
2
0
PPM
0
20
40
60
80
100
120
140
160
180
200
220
240.
PPM

Figure 9

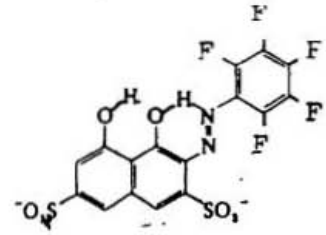
In Figure 9 it can be seen that the signal at 8.10 ppm in the ^{1}H-NMR spectrum is correlated with the carbon signal at 120.01 ppm which is due to C-1 (and C-8) of [15]. The singlet at 4.85 ppm in the ^{1}H-NMR spectrum is correlated with the carbon signal at 26.94 ppm which is due to the methylene-bridge carbon of [15]. The APT shown in the same figure is consistent with the assigned structure.

In light of these findings the observation that the signals due to protons attached to C-1 (and C-8) were diminishing in intensity relative to those of C-3 (and C-6) reported above in Figures 7a-e and 8a-e was confusing. Therefore in order to clarify this it was decided to synthesize a known mono- or disubstituted derivative of chromotropic acid in order to examine its ^{1}H-NMR spectral properties. The mono-phenylazo derivatives Chromotrope 2R [16] (31) and the bis(2-sulfophenylazo) derivative Sulfonazo III [6] (31) are commercially available and their ^{1}H-NMR spectra were recorded. The spectra were complex however, due to the presence of the extra aromatic protons on the phenylazo groups and were not helpful. In order to simplify the ^{1}H-NMR spectrum of Chromotrope 2R [16] an attempt was made at the synthesis of the pentafluoro

analogue [17] using pentafluoroaniline instead of aniline in the synthesis by modification of the procedure reported for [6] (31). However, [17] could not be synthesized and this approach was abandoned.

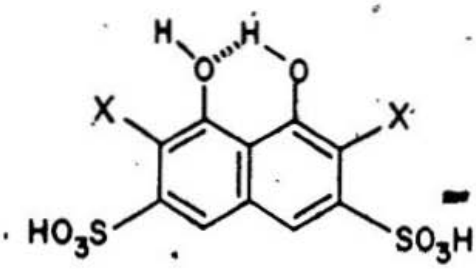


[16]



[17]

The di-iodo, di-bromo and di-chloro derivatives (43) [18a,b,c], appeared to be better choices since there were no additional protons to be considered.



[18a] X = I

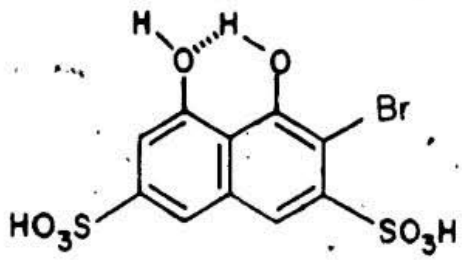
[18b] X = Br

[18c] X = Cl

However, attempts to synthesise these di-halogenated derivatives using the inadequately described procedures that were reported by Maslowska and Duda (43) were unsuccessful. The alternative procedures reported by Kuznetsov and Basargin (47) were then tried. Using their procedure for the synthesis of the monobrominated chromotropic acid [19] afforded only the di-brominated product [18b]. Their procedure was modified and [19] was obtained in approximately 60% yields.

The ^{1}H-NMR spectrum of [18b] was similar to that of [15]. The ^{1}H-NMR spectrum of [19] was more revealing, showing three signals in the aromatic region, one singlet and two doublets.

The two-dimensional ^{1}H-^{13}C chemical shift correlation spectrum of [19] is shown in Figure 10.



[19]

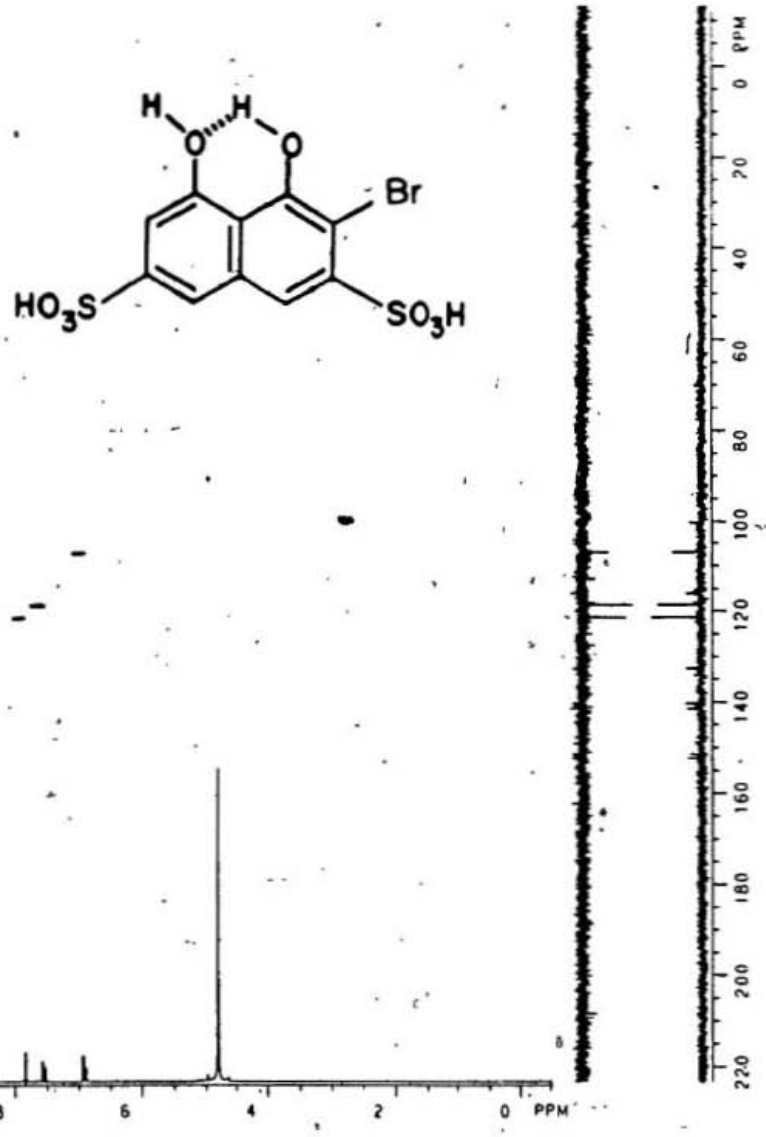

H
H
O
O
Br
HO3S
SO3H
8
6
4
2
0
PPM
0
20
40
60
80
100
120
140
160
180
200
220
PPM

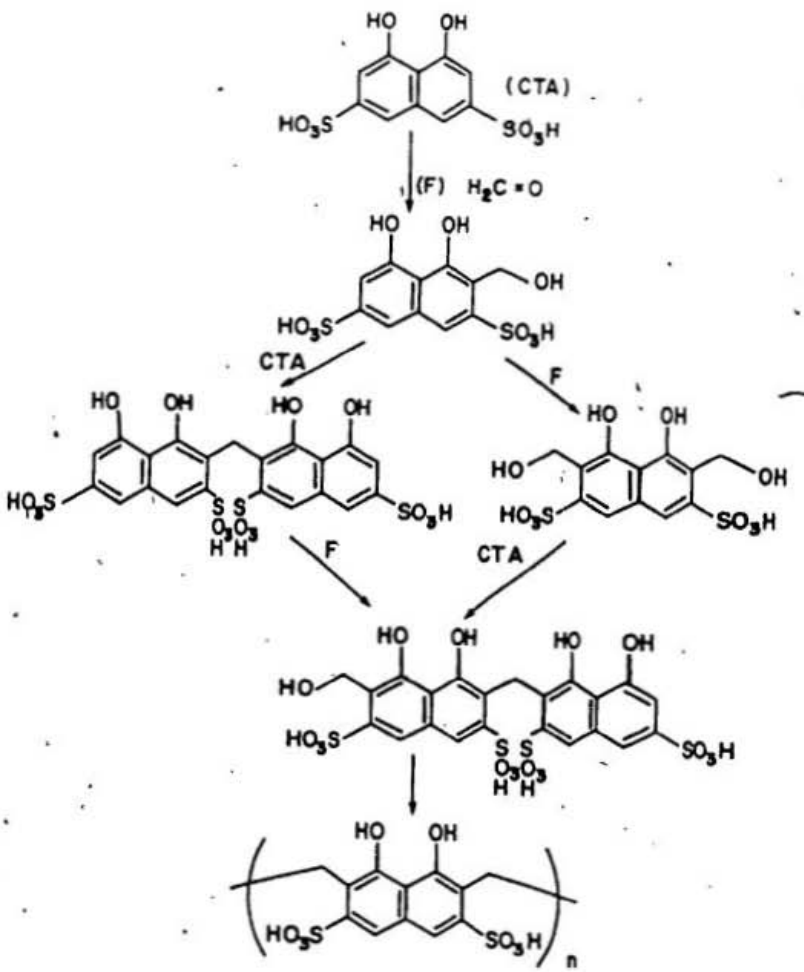
Figure 10

In Figure 10 it can be seen that the singlet at 8.10 ppm in the ^{1}H-NMR spectrum is correlated with the ^{13}C signal at 121.33 ppm which is due to C-1 of [19]. The doublet at 7.80 ppm (J=2Hz) in the ^{1}H-NMR spectrum is correlated with the ^{13}C signal at 118.62 ppm which is due to C-8. The doublet in the proton spectrum at 7.17 ppm (J=2Hz) is correlated with the ^{13}C signal at 107.06 ppm which is due to C-6. The protons on C-6 and C-8 are coupled to each other. The APT shown in the same figure is consistent with the assigned structure.

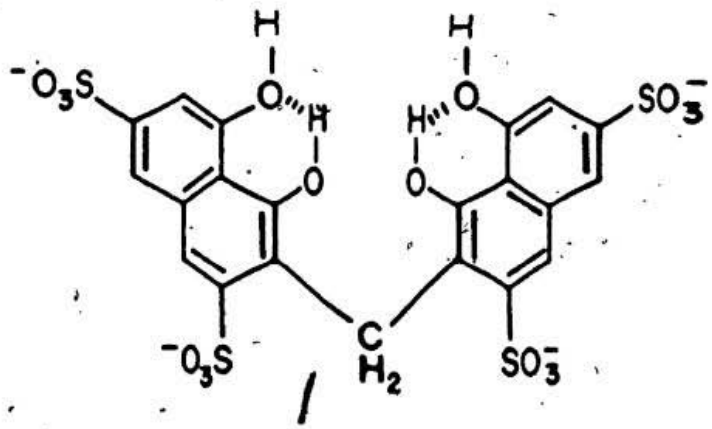
The NMR spectra of [19] provided essential information for interpretation of the spectra obtained earlier and referred to as Figures 7a-e and 8a-e. Figures 8a-e therefore indicate that a monosubstituted chromotropic acid-formaledhyde adduct is being formed in the earlier stages of the reaction. Subsequently, it is converted in the presence of excess formaldehyde into the di-substituted ortho-ortho linear polymer referred to as [15]. Other intermediate adducts as outlined in Scheme 3 could also be formed. Figures 7a-e indicate that after 1.0 h the optimal amounts of mono-substituted adduct could be obtained. Chromotropic acid itself was always present.

Scheme 3

CTA = chromotropic acid; F = formaldehyde.



The reaction could be terminated after approximately 1.0 h by rapidly removing the solvent and any unreacted formaldehyde under high vacuum. Neither TLC nor HPLC was suitable for fractionating and isolating the components of these mixtures because of their extreme water-solubility. Paper chromotography revealed that chromotropic acid was always present but it was not possible to resolve any of the other components of the mixture by this technique. After much experimentation, some of the components of these mixtures could be separated by gel permeation chromatography (48,49) using Sephadex LH-20. The major components that could be identified were chromotropic acid (25-30%) and a reddish coloured compound (55-60%) whose structure is proposed as being [20].



[20]

Numerous attempts at crystallizing compound [20] were unsuccessful. However, clean ^{1}H- and ^{13}C-NMR spectra could be obtained. The two- dimensional ^{1}H-^{13}C chemical shift correlation spectrum of [20] is shown in Figure 11.

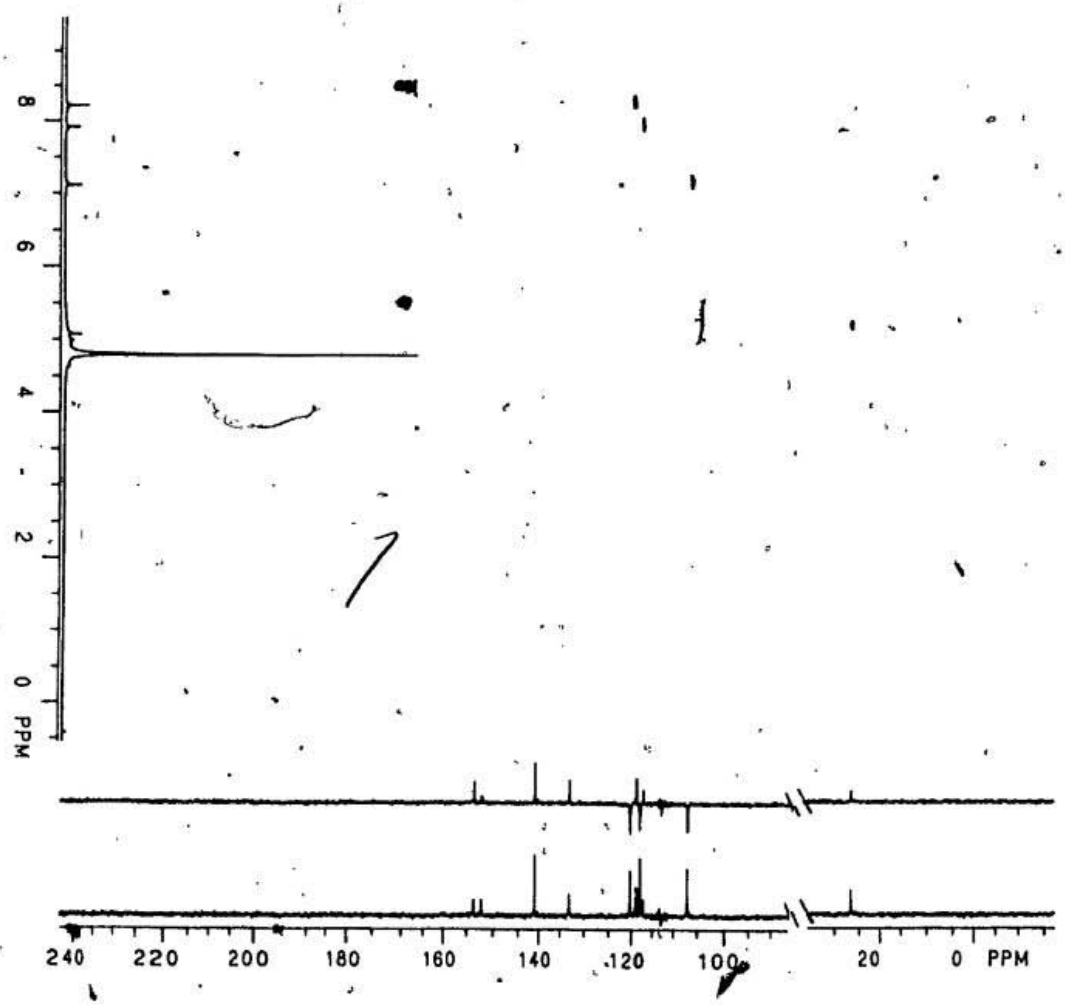


Figure 11

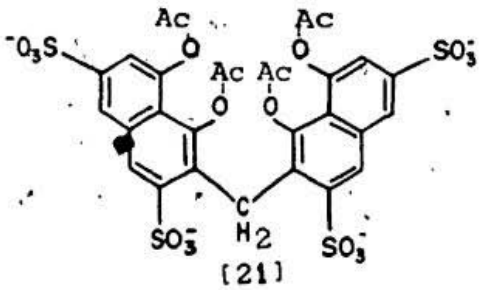
In Figure 11 the singlet at 8.60 ppm in the ^{1}H-NMR spectrum is correlated with the ^{13}C signal at 118.08 ppm which is due to C-8 of [20]. The doublet at 8.30 ppm (J=2Hz) in the ^{1}H-NMR spectrum is correlated with the ^{13}C signal at 120.35 ppm which is due to C-1. The doublet in the proton spectrum at 7.52 ppm (J=2Hz) is correlated with the ^{13}C signal at 106.96 ppm which is due to C-3. The protons on C-1 and C-3 are coupled to each other. The ^{13}C-NMR spectrum however consists of only ten discernable signals. Ideally eleven carbon signals would be expected. Peak height comparisons in ^{13}C-NMR spectra are generally unreliable (39). However, in the series of compounds that have been described above, it is evident that the peak heights of the ^{13}C signals due to C-4 and C-5 (those carbon atoms which are attached to the hydroxyl groups) are very similar to those of the ^{13}C signal of C-2 and C-7 (those carbon atoms which are attached to the sulphonic acid groups). In Figure 11 however, the combined peak heights of the carbon signals at 153.65 and 152.21 ppm assigned to C-4 and C-5 in [20] are approximately one-half of the peak height of the signal at 140.98 ppm. This latter signal whose chemical shift is consistent with the shifts

associated with C-2 and C-7 in chromotropic acid and its derivatives that have been described above is therefore assigned to both carbon atoms C-2 and C-7. The singlet at 5.45 ppm in the ^{1}H-NMR spectrum correlates with the ^{13}C signal at 26.73 ppm which is due to the carbon atom of the methylene bridge between the two chromotropic acid molecules. The APT shown in the same figure is consistent with the assigned structure.

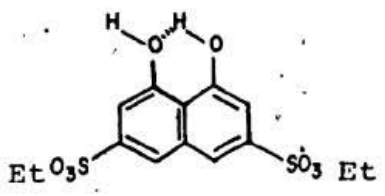
Acetylation of [20] afforded a tetra-acetoxy compound [21] whose ^{1}H-NMR spectrum was similar to that of [20] but which in addition showed clearly two different types of acetate methyl groups. This is additional evidence for the structure proposed for [20].

It should be noted that because of the highly water-soluble nature of chromotropic acid and its adduct(s) with formaldehyde separation and purification had been a major difficulty. Prior to our discovery that Sephadex LH-20 was suitable for the desired separations, many experiments were directed at synthesizing organic-soluble derivatives. The water-solubility of chromotropic acid and similar compounds is due to the sulphonic acid groups (31). The conversion of chromotropic acid to the corresponding

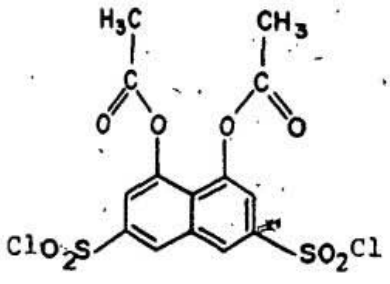
diethyl ester [22] was therefore attempted. The disulphonyl chloride [23] was synthesized by reacting [14] with refluxing phosphoryl chloride. Reaction of [23] with anhydrous ethanol gave the diacetoxy diethyl sulphonyl ester [24]. Careful alkaline hydrolysis of [24] afforded [22]. Spectral data were consistent with the proposed structures. Unfortunately [22] was only soluble in water and highly hydrophilic polar organic solvents and could therefore not be extracted into water immiscible organic solvents. This approach was not pursued further because of the success eventually obtained with the gel permeation chromatography.



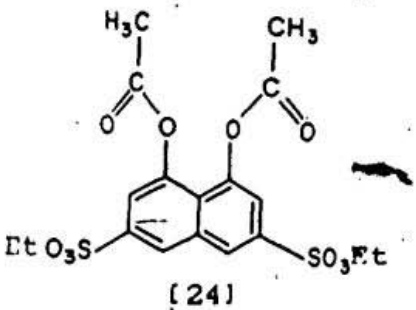
[21]



[22]



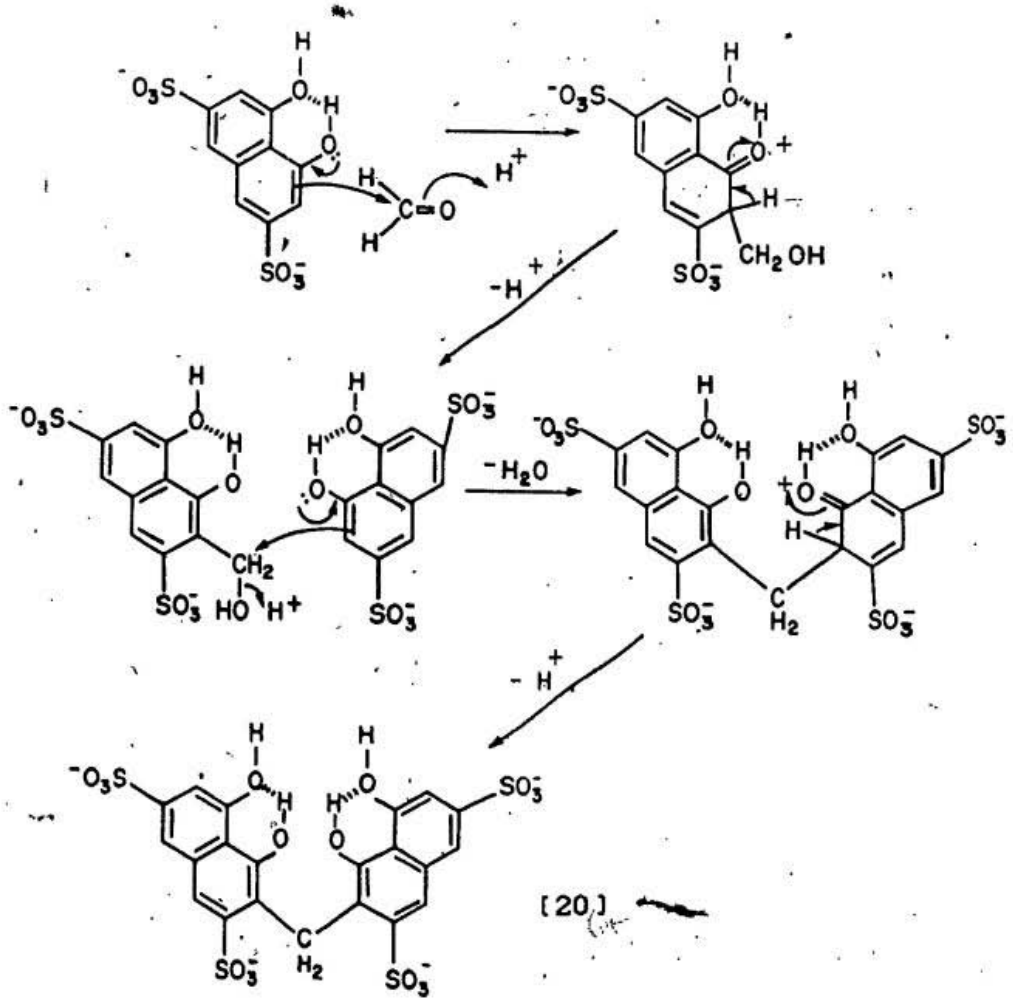
[23]



[24]

Strong evidence that reaction of formaldehyde occurs at C-3 (or C-6) of chromotropic acid has been obtained by the experiments described above. However, it remained to be demonstrated that the adduct [20] is indeed a precursor to the chromogen that is produced in the analytical method. The formation of [20] can be envisioned by the mechanism outlined in Scheme 4:

Scheme 4



CHAPTER 3

The adduct [20] as mentioned above has a red colour and in aqueous solution has an absorbance maximum in the visible range at 560nm. The chromogen formed in the analytical solutions, which are 10.2 M in sulphuric acid, has an absorbance maximum at 580 nm with reported absorptivity values of 8.9×10^3 (10) and 4.6×10^4 M^{-1} cm^{-1} (20). When [20] was dissolved in aqueous 10.2 M sulphuric acid solutions it afforded a solution whose absorbance maximum was also 580 nm. The ^{13}C-NMR spectrum of [20] in these sulphuric acid solutions could not be observed under the conditions used. Under these same conditions however the ^{13}C-NMR of chromotropic acid itself could be observed and recorded.

When oxidation of dilute aqueous solutions of [20] was attempted with 30% aqueous hydrogen peroxide no purple colour formation was observed. Addition of concentrated sulphuric acid to these solutions to bring them up to the equivalent of 10.2 M in sulphuric acid did not produce the purple chromogen either. Therefore it was concluded that a

simple oxidation alone of the adduct did not produce the chromogen.

The effect of acid strength on chromogen formation was then evaluated. Olanski and Deming (33) have determined the optimal absorbance response in the chromotropic acid procedure using simplex optimization. The optimal absorbance response was shown by them to be a function of the volume of concentrated sulphuric acid and the volume of the aqueous chromotropic acid solution. Preliminary experiments indicated to us that the purple chromogen could also be produced using concentrated hydrochloric acid, perchloric acid and 85% phosphoric acid. Calibration lines were determined for each of the above acids in the usual way using standard formaldehyde and 5% aqueous chromotropic acid solutions. These determinations were undertaken in order to evaluate whether more sensitive responses could be obtained using these other acids. Figure 12 shows the calibration lines obtained. Concentrated sulphuric acid gave the best calibration line slope. The calibration line slope obtained with 85% phosphoric acid was only 30% of that obtained with the concentrated sulphuric acid. Glacial acetic acid failed to generate any colour.

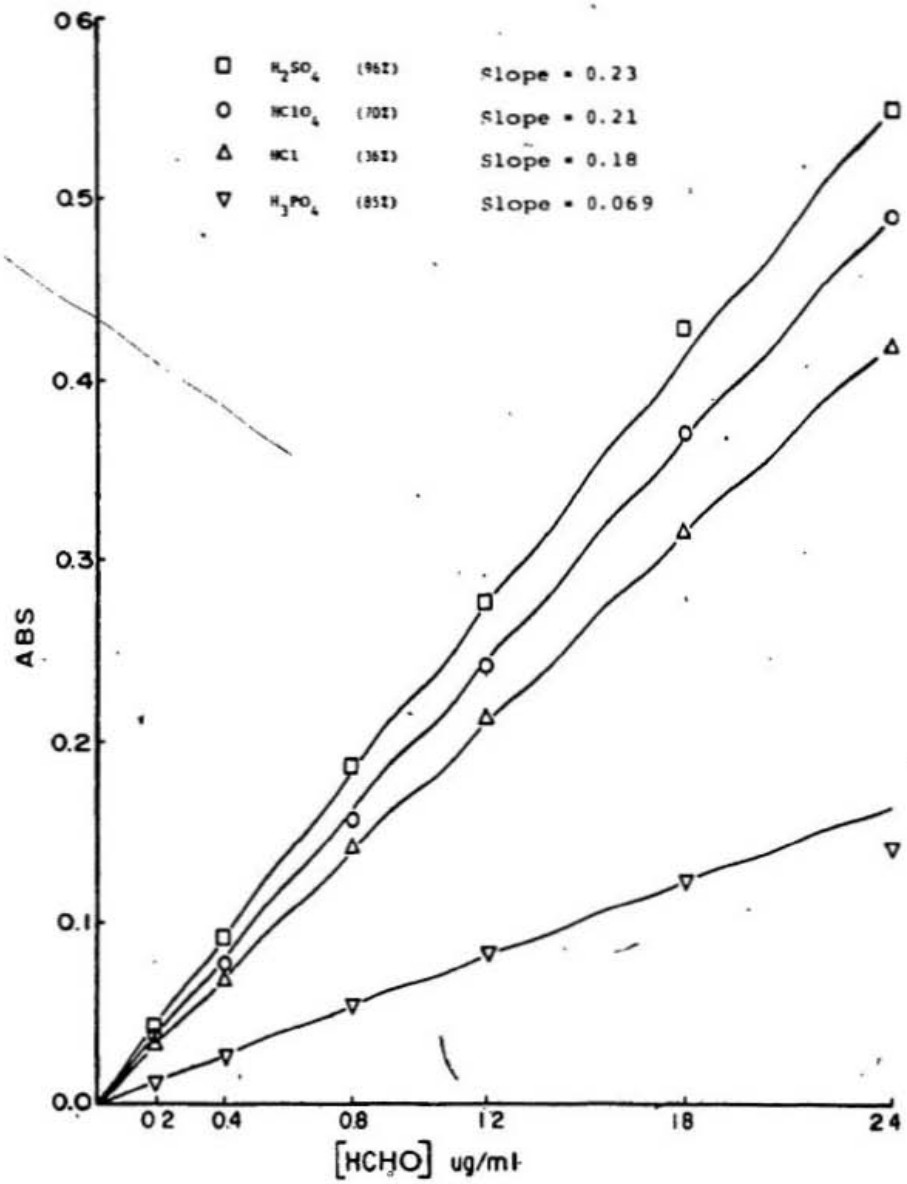

□ H_2SO_4 (96%) Slope = 0.23
○ $HClO_4$ (70%) Slope = 0.21
△ HCl (36%) Slope = 0.18
▽ H_3PO_4 (85%) Slope = 0.069
ABS
0.0
0.1
0.2
0.3
0.4
0.5
0.6
02
04
08
12
18
24
[HCHO] ug/ml

Figure 12

Since sulphuric acid appeared to be the optimal acid to use the effect of using different concentrations was examined. The use of 3.0 mL of concentrated (96%), or 3.0 mL of 38% sulphuric acid were compared in the same way as described above for Figure 12 except that the resulting solutions were all heated for only 1 h. The analytical solutions formed by the above correspond to solutions which are 10.2 M and 4.10 M in sulphuric acid respectively. After heating, only the solutions which were 10.2 M in sulphuric acid had developed the purple chromogen. These solutions were diluted by the addition of water in proportions so that the sulphuric acid concentrations were reduced to 4.10 M. The calibration line slopes derived from these diluted solution were equivalent to those obtained before the dilution. This implied that the chromogen once formed originally with concentrated sulphuric acid could still exist in the 4.10 M sulphuric acid solutions. These results are shown in Figure 13. When concentrated sulphuric acid was added to 4.10 M sulphuric acid solutions the calibration line slopes of these reconstituted solutions were identical to the previously obtained slopes within experimental error. This

result is included in Figure 13 . Thus, proof was obtained that the chromogen formation was not simply pH-dependent either.

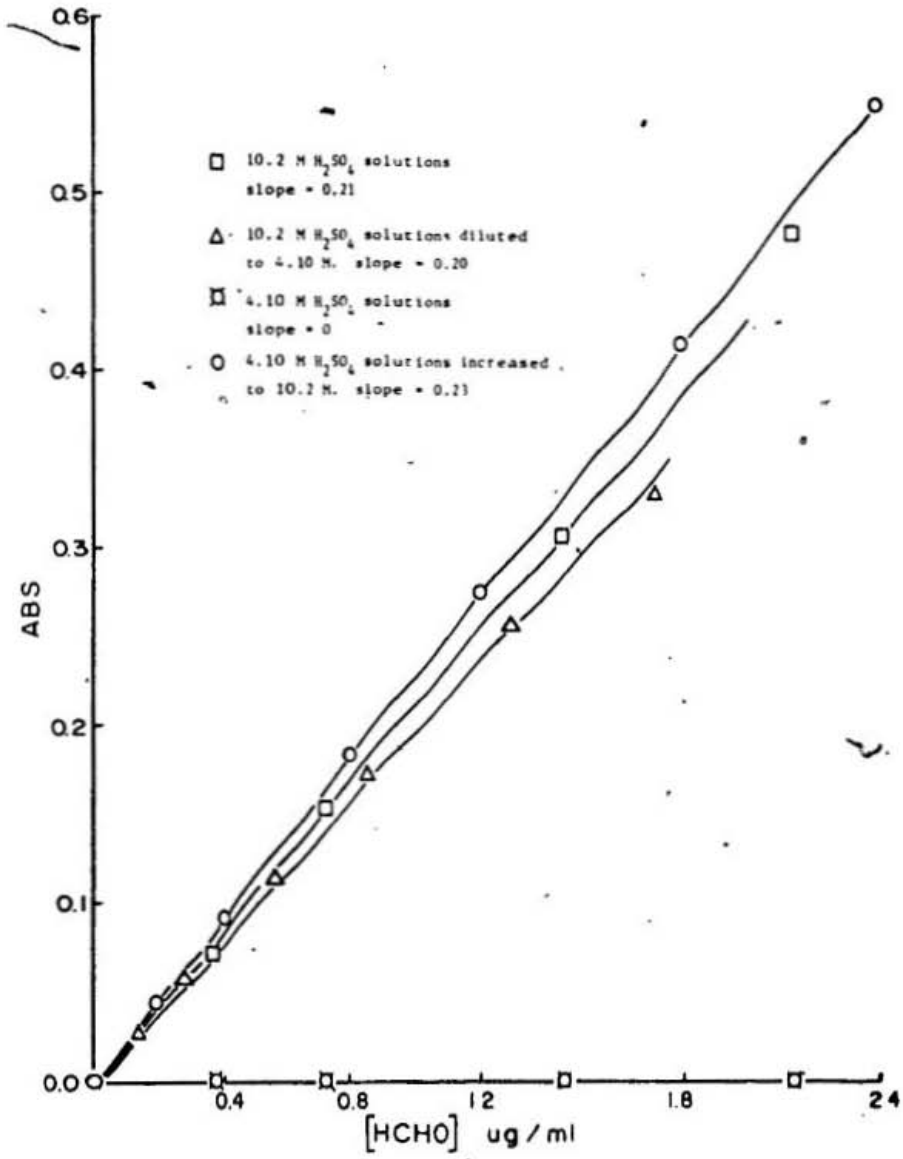


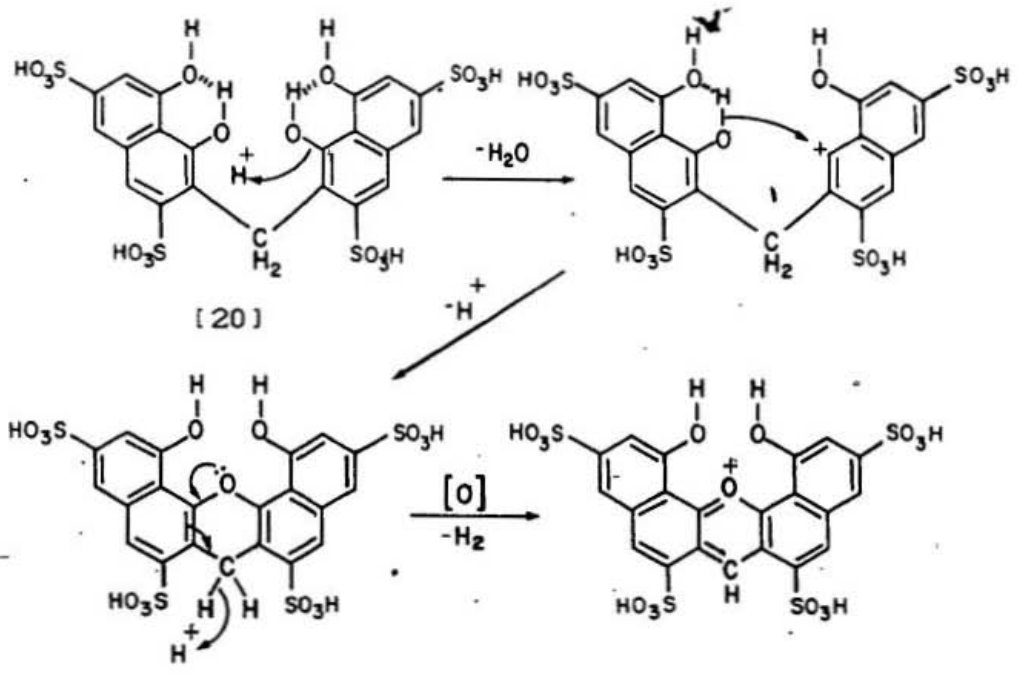
Figure 13

When zinc powder was added to the standard acidic solutions containing the chromogen and whose calibration lines are typified in Figures 13 the chromogen was completely destroyed. However, upon merely re-heating these solutions the chromogen could be regenerated and the original calibration lines were obtained. Thus it appeared as though that the chromogen formation involved an oxidation step which occurs in the concentrated sulphuric acid. The chromogen once formed can be reversibly reduced by the addition of a metal reducing agent such as zinc.

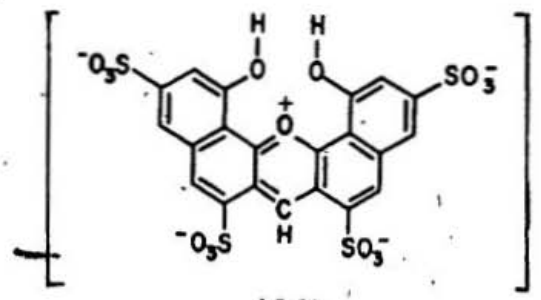
All of the above evidence is consistent with a mono-cationic dibenzoxanthylium structure for the chromogen and not the para,para-quinoidal one that is commonly cited. Thus, the experimental data suggests that the first step in the analytical reaction is the formation of an adduct such as [20]. The second step involves the dehydration by a strong acid to form the dibenzoxanthene-type compound [25]. The oxidation of such a compound would be favoured since it can produce the mono-cationic dibenzoxanthylium structure [9]. This structure satisfies the Huckel (4n+2) rule for aromaticity since it has 22 pi-electrons and is planar as revealed by molecular models.

Scheme 5 outlines these steps.

Scheme 5



[20] [25] [9]



[26]

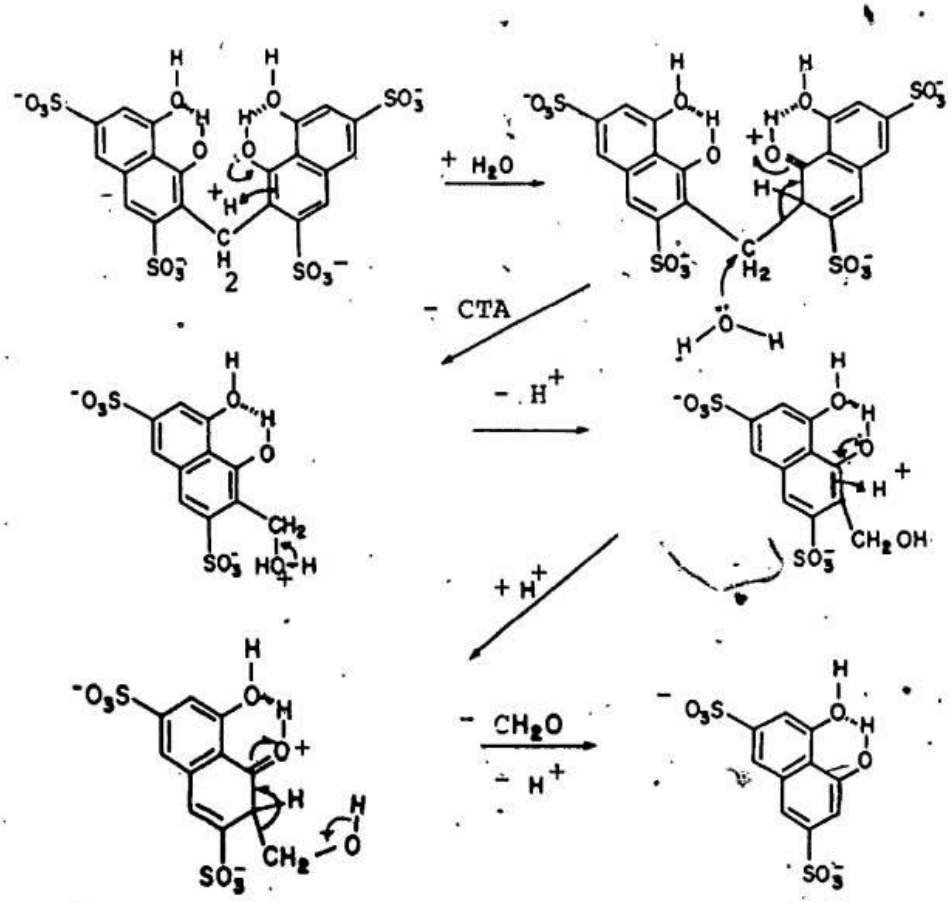
Several experiments were conducted using tetra-n-butylammonium phosphate in order to extract [26] the quaternary ammonium salt of the putative chromogen [9] directly from the acidic solutions. It was possible to do this using dichloromethane as the solvent. The expected aromatic region protons in the ^{1}H-NMR spectrum at room temperature were not detected, however.

It was not practical to attempt to isolate the chromogen directly from the acidic solutions by gel permeation chromatography using Sephadex LH-20 since the gel itself undergoes charring when exposed to the strongly acidic solutions. If a solution containing the chromogen is first neutralized by the addition of sodium hydroxide, and the resulting solution evaporated to dryness, the relatively large amounts of sodium sulphate produced interfere with the separation of the smaller amount of chromogen present. By removing most of the sodium sulphate first by repeated crystallization and filtration, a residue could finally be obtained which could be fractionated using Sephadex LH-20. All that could be identified however, was chromotropic acid and the adduct [20].

The chromogen could apparently also be produced using concentrated hydrochloric acid as was demonstrated above in Figure 12. The reaction of adduct [20] with concentrated hydrochloric acid also afforded a purple chromogen whose visible spectrum was similar to that which is obtained in the analytical reactions. It was reasoned therefore that after the reaction of adduct [20] with concentrated hydrochloric acid was effected, the chromogen could be isolated by merely evaporating the solvent containing the hydrochloric acid. The same would not be possible to achieve with sulphuric acid. When such an experiment was carried out in concentrated hydrochloric acid, the residue obtained after the chromogen had apparently been formed was chromatographed on Sephadex LH-20. Again, only chromotropic acid and the adduct [20] could be isloated. The presence of chromotropic acid was of concern since chromatographically pure adduct [20] was used in the reaction. An experiment was conducted in which a solution of [20] in water in an NMR tube was heated in a boiling water bath. The 60 MHz ^{1}H-NMR spectrum of the mixture was periodically monitored. After 3 h it was found that approximately 50% chromotropic acid was now present

in the aqueous solution. Thus, it appears as though the formation of [20] is reversed in water. It is likely therefore that the chromotropic acid observed in the two previously described experiments resulted from the retro-addition in water of the formaldehyde from [20]. Scheme 6 outlines this possibility.

Scheme 6



All attempts at isolating and identifying the putative chromogen [9] or its precursor [25] had not succeeded to this point. A final experiment was undertaken in order to show indirectly that the adduct [20] was indeed the precursor of [9] via the dibenzoxanthene-type compound [25]. Thus, an aqueous 1.0×10^{-4} M solution of [20] was prepared. From this solution, five aqueous solutions were prepared by appropriate dilution. Assuming that there is one mole of formaldehyde in each mole of the adduct [20], the five solutions which were prepared had formaldehyde equivalent concentrations in the range normally employed in the analytical calibration line determinations. Two-mL aliquots of these solutions were treated with 300 uL of deionized organic-free water and 3.0 mL of concentrated sulphuric acid in a manner exactly analogous to the NIOSH procedures. As a control, the usual standard formaldehyde calibration line was also determined using 300 uL of 5% aqueous chromotropic acid and 3.0 mL of concentrated sulphuric acid, exactly according to the NIOSH procedures at the same time. The calibration lines were determined for the respective resultant solutions which were obtained after 1 h heating. The slopes were found to be different. A

slope of 0.23 AU/(ug/mL) was determined for the control solutions, and a slope of 0.096 AU/(ug/mL) for those prepared from the adduct.

Careful re-evaluation of the results obtained from this experiment indicated that an inappropriate comparison was being made. Since the analytical solutions normally contain a large excess of chromotropic acid over the formaldehyde that is determined, it was reasoned that a true comparison between the absorbances measured from the solutions derived from [20] could only be made if the 300 uL of 5% aqueous chromotropic acid was also present. Therefore, a replicate set of the five solutions prepared previously from the aqueous solution of [20] was prepared and each solution was treated as before except that each now contained 300 uL of 5% aqueous chromotropic acid instead of the 300 uL amount of water. The calibration lines were determined again for the resultant solutions which were obtained after heating for 1 h. The slope obtained was now identical to that of the control. These results are presented in Figure 14 .

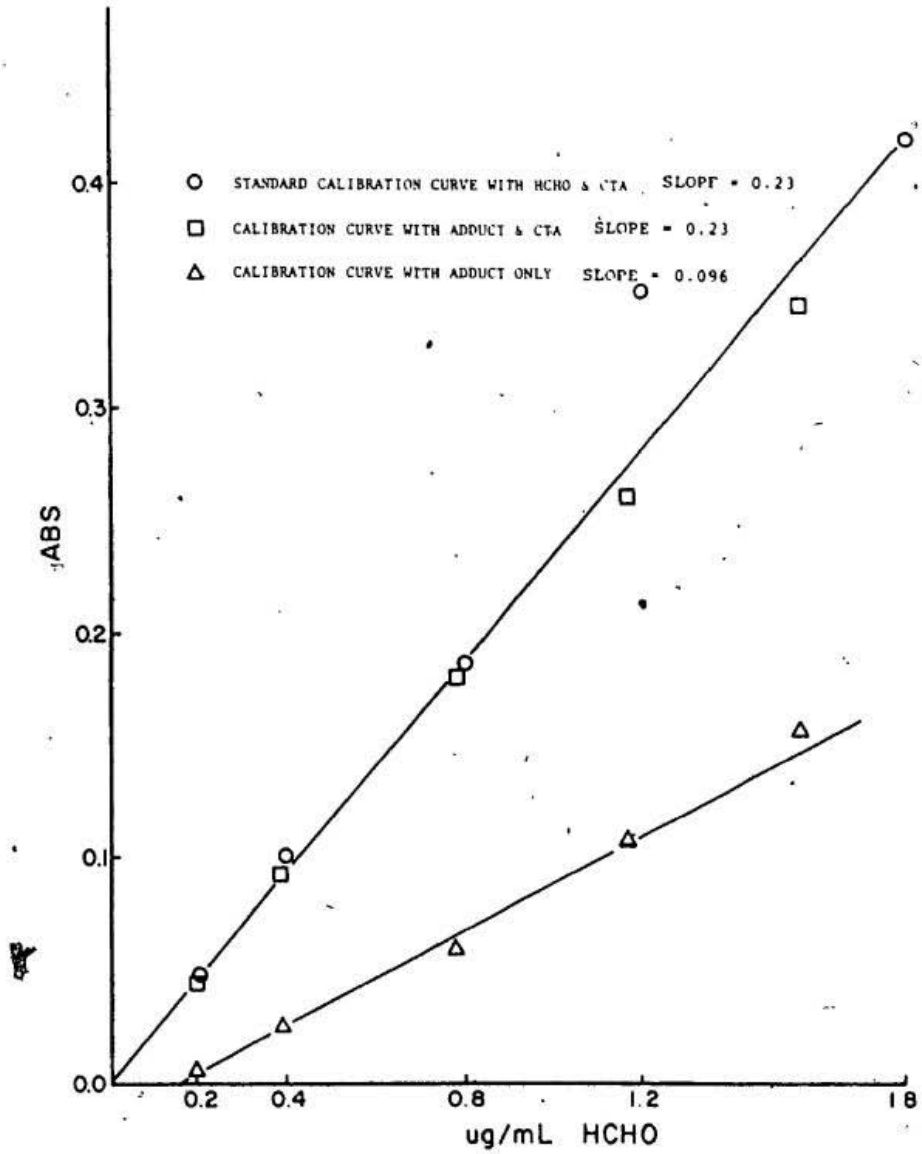
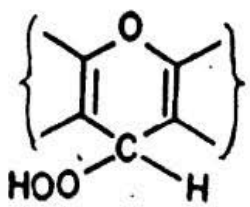

STANDARD CALIBRATION CURVE WITH HCHO & CTA SLOPE = 0.23
CALIBRATION CURVE WITH ADDUCT & CTA SLOPE = 0.23
CALIBRATION CURVE WITH ADDUCT ONLY SLOPE = 0.096
ABS
0.0
0.1
0.2
0.3
0.4
0.2
0.4
0.8
1.2
1.8
ug/mL HCHO

Figure 14

The results obtained in these last two experiments are also consistent with the mechanism outlined in Scheme 6. Further, the calibration line observations provide additional evidence that an equilibrium reaction exists in which the excess chromotropic acid shifts the equilibrium favourably toward the direction of the optimal chromogen formation. From the higher slope obtained in these experiments the absorptivity for the chromogen was calculated to be 1.8×10^{4} M^{-1} cm^{-1} which is two- to four-fold greater than those values reported previously (10,20).

The fact that oxidation of [20] by hydrogen peroxide did not afford the chromogen could be due to the fact that since it was in a large excess, the former can undergo addition to the double bond leading to a "leuco"-type structure (50) such as [27].



[27]

CONCLUSIONS

1. The site of the reaction of formaldehyde with chromotropic acid in aqueous or aqueous acidic conditions occurs on the carbon atom which is ortho- to both the hydroxyl and sulphonic acid groups. This has been proven by the use of a variety of ^{1}H- and ^{13}C-NMR experiments.

2. Using gel permeation chromatography on Sephadex LH-20 the water-soluble components of the synthetic reactions employing chromotropic acid and formaldehyde could be separated.

3. An adduct containing a methylene bridge formed by the coupling of a single molecule of formaldehyde between two molecules of chromotropic acid could be isolated and characterized by ^{1}H- and ^{13}C-NMR spectroscopy. Attempts at crystallization of this adduct were unsuccessful.

4. The adduct undergoes a retro-addition equilibrium in aqueous solution.

5. The adduct was demonstrated to be a precursor of the same chromogen that is observed in the NIOSH

analytical method, by calibration line studies. The chromogen could be isolated but could not be characterized.

6. The formation of the chromogen is not simply a function of pH alone. It is hypothesised that a dehydration step is first required to produce a dibenzoxanthene-type compound. This compound can then undergo facile oxidation to produce the corresponding mono-cationic dibenzoxanthylium structure. The mono-cation is planar and obeys the Huckel (4n+2) rule for aromaticity.

7. The often-quoted para,para-quinoidal structure for the chromogen appears to be very unlikely.

8. Concentrated sulphuric acid appears to be the optimal acid to use in the method.

9. Simple modifications to the procedure do not seem feasible in light of the understanding of the reaction gained during this study.

EXPERIMENTAL

Melting points were determined on a Fisher-Johns melting point (hot stage) apparatus.

^{1}H-NMR spectra were recorded with the following instruments at the designated frequencies: Varian Anaspect EM 360 at 60 MHz, Bruker WP-80 at 80 MHz, or GE GN-300NB at 300 MHz. ^{13}C-NMR spectra were recorded with the Bruker WP-80 at 20.1 MHz, and/or the GE GN-300NB at 75.47 MHz. The solvent used was either deuterium oxide or water as noted in the experimental details. Ultraviolet and visible spectra were determined on a Unicam SP.800 Ultraviolet spectrophotometer. Calibration lines were determined with a Perkin-Elmer Hitachi-Coleman 139 grating single-beam spectrophotometer. Infrared spectra were recorded on a Perkin-Elmer 1320 infrared spectrophotometer using KBr disks.

Sephadex LH-20 as supplied by Pharmacia Fine Chemicals was used in the gel permeation chromatography. Merck silica gel was used for preparative-layer chromatography using 20 x 20 cm plates of 0.75 mm

thickness. Synthetic reagents purchased from Aldrich Chemical Co. were used except where otherwise noted.

Crystal and molecular structure analyses were conducted on an Enraf Nonius CAD-4 four circle diffractometer using the omega scan mode at Energy, Mines and Resources Canada, Booth Street, Ottawa.

Purification of 4,5-dihydroxy-2,7-naphthalenedisulphonic acid disodium salt [4].-The material supplied by Sigma Chemical Co., was specified as being only 98% pure. Purified [4] was obtained by dissolving 20 g of the Sigma product in 100 mL of water in a 125-mL Erlenmeyer flask on a hot plate. A solution of 20 g of sodium chloride in 50 mL of hot water was added to the chromotropic acid solution until a precipitate formed. The flask was stoppered and the solution was allowed to first crystallize at room temperature and then at low temperature in a refrigerator. The crystals were filtered with suction and dried in air. The product was almost colourless: ^{1}H-NMR (D_2O) δ ppm: 7.18 (d, J=2Hz, 1H, Ar-H), 7.88 (d, J=2Hz, 1H, Ar-H); ^{13}C-NMR and APT (D_2O) δ ppm: 106.44 (C-3 and C-6, one attached proton), 116.18 (C-10; zero attached protons), 118.12 (C-1 and C-8, one attached proton), 134.64 (C-9, zero attached protons), 141.36 (C-2 and C-7, zero attached protons), 153.76 (C-4 and C-5, zero attached protons); 2-D ^{1}H-^{13}C correlation (D_2O): 7.18 ppm correlates with 106.44 (C-3 and C-6), 7.88 ppm correlates with 118.12 (C-1 and C-8); uv (H_2O) λ_{max} absorbances: 316, 332, 346 nm; ir (KBr) γ_{max}: 3,500 cm^{-1} (-SO_2O-H and ArO-H), 1,200 cm^{-1} (S=O).

Deuterium exchange experiments with chromotropic acid.-In a typical experiment, an NMR tube was charged with a solution containing 67 mg of purified [4] in 0.30 mL of deuterium oxide. A small amount of DSS as internal standard was added to the solution. The NMR tube was heated in a boiling water bath. Periodically, after every ten minutes' heating the ^{1}H-NMR spectra were recorded until the signal at 7.18 ppm completely disappeared and the signal at 7.88 ppm collapsed to a sharp singlet. The deuterium exchange could be reversed by adding 0.1 mL of water to the NMR tube and thoroughly mixing the mixture. The ^{13}C-NMR spectra were recorded for a freshly prepared deuterium oxide solution of purified [4], and following heating of the NMR tube in a boiling water bath for 15 min. Side-by-side comparison of the spectra indicate that the intensity of the 106.44 ppm signal (C-3 and C-6) was reduced by over 90%. All other signals were unchanged.

4,5-Diacetoxy-2,7-naphthalenedisulphonic acid disodium salt [14].- In a 25-mL Erlenmeyer flask, 1.0 g (2.68 mmol) of chromotropic acid disodium salt was dissolved into 3.0 mL of 3.0 M aqueous sodium hydroxide. To this solution was

added approximately 2 g of crushed ice, followed by 0.6 mL of acetic anhydride. The mixture was stirred for 5-10 min with ice-bath cooling. The diacetate separated out as colourless crystalline needles. It was filtered by suction filtration, washed with portions of absolute ethanol and dried in air. Recrystallization from water afforded 0.52 g (42%) of crystals having decomposition point at 320°C. X-Ray crstallographic analysis revealed that the compound was a dihydrate: ^{1}H-NMR (D_2O) δ ppm: 2.43 (s,3H,CH_3), 7.72 (d, J=2Hz, 1H, Ar-H), 8.52 (d, J=2Hz, 1H, Ar-H): uv (H_2O) λ_{max} absorbances: 315, 329, 339 nm

Crystal and molecular stucture of 4,5-Diacetoxy-2,7-naphthalenedisulphonic acid disodium salt [14]:-The crystal and molecular structure of [14] were determined by single crystal X-Ray diffraction. Diffraction data were obtained from a crystal (0.1 x 0.15 x 0.25 mm) mounted on an Enraf Nonius CAD-4 four circle diffractometer using the omega scan mode. The crystal is monoclinic, space group $P2_1/m$. The unit cell dimensions were obtained from 21 reflections with $67.00° < 2\theta < 97.00°$ and are a=5.67839(7), b=22.2973(2), c=7.8438(1) Å, β=95.644(2); Z=2; d_{calc}=1.626 Mg m^{-3}.

Diffraction data were collected with graphite monochromatized MoKα_1 radiation (λ=0.70930 Å, μ=0.35 mm^{-1}) to $2\theta_{max}$=49.8°. Of the 2,549 refections which were measured, 1,768 were unique, and 1,411 were considered significant ($I_{net.} > 2.5\sigma(I_{net.})$). No corrections were made for absorption. The structure was solved by direct methods and refined by least squares using the NRCVAX CRYSTAL suite of programmes (51). Hydrogen atoms were found from difference Fourier maps. All atoms, except hydrogen, were allowed to refine anisotropically. In the last difference map the deepest hole was -0.290 e Å^{-3} and the highest peak was +0.400 e Å^{-3}. The final residuals were R=0.037, wR=0.027, goodness-of-fit=2.429 for significant reflections, and R=0.048, wR=0.028 for all reflections. The maximum shift/sigma ratio was 0.259.

$$R = \Sigma (|F_o| - |F_c|) / \Sigma (|F_o|)$$

$$wR = \sqrt{[\Sigma w(|F_o| - |F_c|)^2 / \Sigma (w|F_o|^2)}$$

$$\text{Goodness-of-fit} = \sqrt{[\Sigma w(|F_o| - |F_c|)^2 / (\text{No. of reflections} - \text{No. of parameters})}$$

Preparation of adduct [15].-The 1:1 formaldehyde-chromotropic acid adduct was prepared exactly in the same way as decsribed for [20] except that equimolar amounts of

[4] and formaldehyde were used. The resulting cherry-red reaction mixture was chromatographed on Sephadex LH-20 in the same manner as for [20]. Only one major component was isolated whose spectral properties was consistent with the structure [15]: ^{1}H-NMR (D_2O) δ ppm: 8.10 (s, C_{α}- and C_{γ}-protons), 4.85 (s, methylene protons); ^{13}C-NMR (D_2O) δ ppm: 26.94 (C-11, methylene bridge carbon), 117.48 (C-10), 119.28 (C-3 and C-6), 120.01 (C-1 and C-8), 130.45 (C-9), 140.03 (C-2 and C-7), 151.52 (C-4 and C-5); uv (H_2O) λ_{max} absorbances: 320, 337, 353 nm.

In-situ ^{1}H-NMR studies of the reaction with 1:2 and 1:1 molar ratios of formaldehyde and chromotropic acid [4].- A three-necked round-bottom flask was equipped with a magnetic stirring bar, a reflux condenser and a septum through which a slight positive pressure of Argon was introduced and maintained. To the flask was added 1.0 g of [4] in 15 mL of deionized organic-free water. Either 1.0 or 2.0 mL of aqueous 1.32 M methanol-free formaldehyde was added and the mixture refluxed. One-mL aliqouts were periodically removed via a syringe and were evaporated to dryness on a rotatory evaporator and on a vacuum pump. The

residues were each redissolved in 0.3 mL of D_2O and their 60 MHz NMR spectra were recorded.

3,6-Dibromo-4,5-dihydroxy-2,7-naphthalenedisulphonic acid disodium salt [18b].- In a 25-mL Erlenmeyer flask 1.9 g (5.1 mmol) of chromotropic acid disodium salt was mixed with 12 mL of concentrated sulphuric acid (96%, d=1.84 g/mL). The mixture was heated and stirred at 80°C until all the solid dissolved. The solution was cooled to approximately 15°C and with continuous cooling and stirring, a solution of 0.8 g (5.0 mmol) of bromine in 3.0 mL of glacial acetic acid was added dropwise over 1 h. After the addition of bromine, the solution was stirred and cooled for an additional 3 h. To this solution, approximately 15 g of ice was added. The white precipitate was filtered off with suction, using a sintered-glass funnel. The residue was dissolved in a minimal amount of water at room temperature. To this aqueous solution one-half of its volume of concentrated hydrochloric acid solution was added. The greyish white leaflets were once again filtered off using suction and a sintered glass funnel, and were press-dried. The residue was further dried

under vacuum to afford 1.25 g (46%) of [18b] which decomposed above 40°C with liberation of bromine: ^{1}H-NMR (D_2O) δ ppm: 8.10 (s, Ar-H); ^{13}C-NMR (D_2O) δ ppm: 102.44 (C-3 and C-6), 116.26 (C-10), 122.04 (C-1 and C-8), 130.74 (C-9), 140.11 (C-2 and C-7), 149.62 (C-4 and C-5); 2-D ^{1}H-^{13}C correlation (D_2O), δ 8.10 ppm correlates with 122.04 (C1 and C-8); APT (D_2O) 122.04 ppm (C-1 and C-8, one attached proton); uv (H_2O) λ_{max} absorbances: 340, 358, 375 nm.

3-Bromo-4,5-dihydroxy-2,7-naphthalenedisulphonic acid disodium salt (19).- In a 25-mL Erlenmeyer flask 1.9 g (5.1 mmol) of chromotropic acid disodium salt was mixed with 12 mL of concentrated sulphuric acid (96%, d=1.84 g/mL). The mixture was heated and stirred at 80°C until all the solid dissolved. The solution was cooled to approximately 15°C and with continuous cooling and stirring, a solution of 0.4 g (2.5 mmol) of bromine in 3.0 mL of glacial acetic acid was added dropwise over 3 h. After the addition of bromine, the solution was stirred and cooled for an additional 1 h. To the solution, approximately 15 g of ice was added. The white precipitate was filtered off with suction, using a

sintered glass funnel. The residue was dissolved in a minimal amount of water at room temperature. To this aqueous solution one-half of its volume of concentrated hydrochloric acid solution was added. The greyish white leaflets were once again filtered off using suction and a sintered glass funnel, and were press-dried. The residue was further dried under vacuum to afford 1.4 g (61%) of [19] which decomposed at 40~C with liberation of bromine: ^{1}H-NMR (D_2O) δ ppm: 7.17 (d, J=2Hz, 1H, Ar-H), 7.80 (d, J=2Hz, 1H, Ar-H); 8.10 (s, 1H, Ar-H); ^{13}C-NMR (D_2O) δ ppm: 100.51 (C-3), 107.06 (C-6), 115.96 (C-10), 118.62 (C-8), 121.33 (C-1), 132.63 (C-9), 140.17 (C-7), 141.28 (C-2), 151.35 (C-4), 152.23 (C-5); uv (H_2O) λ_{max} absorbances: 316, 336, 348 nm.

Preparation of adduct [20].-In a three-necked flask equipped with a magnetic stirring bar and a dropping funnel containing 0.87 mL (1.1 mmol) of aqueous 1.32 M methanol-free formaldehyde solution, was placed a solution of 876 mg (2.04 mmol) of purified [4] in 10.0 mL of deionized organic-free water. The reaction mixture was maintained under a slightly positive pressure of Argon. The

solution was slowly heated and maintained at refluxing temperature and the formaldehyde solution was added dropwise over 40 min. After the addition of the formaldehyde was completed the mixture was refluxed for a further 20 min. The reaction mixture was cooled to room temperature and evaporated to approximately 6 mL on a rotatory evaporator. This reaction concentrate was fractionated on a 2.0 cm x 25 cm open glass column packed with 20 g Sephadex LH-20 gel in water. The eluting solvent used was deionized organic-free water. The flow rate was 2 mL/min. Approximately thirty 10-mL fractions were collected. The fractions were examined by uv spectroscopy and those having similar absorbances were combined and evaporated to dryness on the rotatory evaporator and under high vacuum. Two components were isolated and characterized. Fractions 10-12 contained 235 mg (27%) of a product whose ir, uv-vis and ^{1}H-NMR spectra and R_f on paper chromatography was identical to those of [4]. Fractions 13-16 contained 475 mg (55%) of [20]: ^{1}H-NMR (D_2O) δ ppm: 5.03 (s, methylene protons), 7.00 (d, J=2Hz, 1H, C_4-H), 7.83 (d, J=2Hz, 1H, C_1-H): 8.17 (s, 1H, C_8-H); ^{13}C-NMR (D_2O) δ ppm: 26.73 (C-11, methylene bridge carbon), 106.96

(C-6), 117.26 (C-10), 118.08 (C-8), 118.96 (C-3), 120.35 (C-1), 132.94 (C-9), 140.98 (C-2 and C-7), 152.21 (C-5), 153.65 (C-4); uv (H_2O) λ_{max} absorbances: 320, 337, 353 nm; ir (KBr) γ_{max}: 3,500 cm^{-1} (-SO_2O-H and ArO-H), 1,200 cm^{-1} (S=O).

Preparation of [21], the tetra-acetate of adduct [20].-In a 10-mL Erlenmeyer flask, 37 mg (5.0×10^{-5} mol) of adduct [20] was dissolved in 0.033 mL of 6.1 M aqueous sodium hydroxide solution. The mixture was then cooled to 0°C in an ice bath. To this ice-cold solution 0.019 mL (2.0×10^{-4} mol) of acetic anhydride was added with stirring. The resultant pale reddish solution was allowed to stir with cooling for 5 min. The sodium acetate produced in the reaction was eliminated by precipitating it as insoluble silver acetate with a solution 34 mg of silver nitrate in 1.0 mL of water. After the insoluble silver acetate was filtered off the filtrate was then concentrated down to approximately 0.6 mL with a rotatory evaporator. The concentrate was then fractionated on a 10 cm x 1 cm open glass column packed with approximately 0.7 g of Sephadex LH-20 gel in water. The eluate was collected as thirty

0.5-mL fractions. The fractions were examined by uv spectroscopy and those having similar absorbances were combined and evaporated to dryness on a rotatory evaporator and under high vacuum. Seven milligrams of a product whose spectral properties were consistent with the structure [21] was obtained: ^{1}H-NMR (D_2O) δ ppm: 1.97 (s, 6H), 2.08 (s, 6H), 5.13 (s, methylene protons, 2H), 7.63 (d, J=2Hz, 2H, C_3-H), 8.47 (d, J=2Hz, 2H, C_1-H); 8.73 (s, 2H, C_4-H); uv (H_2O) λ_{max} absorbances: 319, 334, 356 nm.

Diethyl-4,5-dihydroxy-2,7-naphthalenedisulphonate [22]. -To 20 mg of [24] in 1.0 mL of dry dichloromethane in a 10-mL Erlenmeyer flask, approximately 0.05 mL of 6.0 M aqueous sodium hydroxide was added. After TLC of the mixture indicated that no starting material remained, the mixture was evaporated to dryness on a rotatory evaporator and under high vacuum. The residue (18 mg) was soluble only in ethanol, methanol or water. The ^{1}H-NMR (CD_3OD) spectrum is consistent with the structure [22]: δ ppm: 0.83 (t, J=7.2 Hz, 3H, $-OCH_2\underline{CH_3}$), 3.62 (q, 3H, J=7.2 Hz, 2H, $-O\underline{CH_2}CH_3$), 6.23 (d, J=2Hz, 1H, C_3- and C_4-protons), 7.03 (d, 1H, C_4- and C_1-protons).

4,5-Diacetoxy-2,7-naphthalenedisulphonyl chloride [23].- Two grams of the diacetate [14] was added to 9.0 mL of phosphoryl chloride in a dry 25-mL round-bottomed flask equipped with a stirring bar and a condenser fitted with a drying tube. The mixture was refluxed for approximately 4 h and then cooled to room temperature. The mixture was then slowly poured onto 10 g of crushed ice in a 50-mL beaker, and stirred vigorously. After the excess phosphoryl chloride was hydrolysed, the mixture was transferred to a 60-mL separatory funnel. The solid residue was extracted twice with 25-mL portions of dichloromethane. The organic layers were combined and were washed twice with aqueous 0.5 M hydrochloric acid, followed by drying over anhydrous sodium sulphate. After filtration and evaporation to dryness, 1.4 g (73%) of a residue was obtained whose spectral properties were consistent with the structure [23]: 300 MHz ^{1}H-NMR ($CDCl_3$) δ ppm: 2.50 (s, 3H, acetoxyl methyl protons), 7.92 (d, J=2Hz, 1H, C_3- and C_6-protons), 8.71 (d, J=2Hz, 1H, C_8- and C_1-protons).

Diethyl-4,5-diacetoxy-2,7-naphthalenedisulphonate [24]. -In a 10-mL round-bottomed flask equipped with a strirrer bar

and a rubber septum was suspended 77 mg (0.175 mmol) of the disulphonyl chloride [23] in 2.0 mL of absolute ethanol (40). The mixture was maintained at ice-bath temperature. To this ice-cold suspension was added 0.2 mL anhydrous pyridine. The yellow mixture was stirred at ice-bath temperature for an additional 30 min. The reaction mixture was then transferred to a separatory funnel and partitioned between dilute hyrochloric acid and dichloromethane. The organic phase was washed with an equal volume of water, and dried over sodium sulphate. After filtration and evaporation to dryness, 66 mg of a residue was obtained. Purification of the mixture on a 20 x 20 cm preparative-layer plate (silica gel, 1:9 ether: benzene) to afford 13 mg (16%) of a compound whose spectral properties were consistent with the structure [24]: 300 MHz ^{1}H-NMR ($CDCl_3$) δ ppm: 1.35 (t, J=7.2 Hz, 3H, $-OCH_2\underline{CH_3}$), 2.47 (s, 3H, acetoxyl methyl protons), 4.24 (q, J=7.2 Hz, 2H, $-O\underline{CH_2}CH_3$), 7.73 (d, J=2Hz, 1H, C_3- and C_6-protons), 8.53 (d, 1H, C_8- and C_1-protons).

Calibration line determinations.

a). Modified NIOSH procedure.-The basic NIOSH procedure was followed for the determination of calibration lines for standard formaldehyde solutions except that the following modifications were incorporated into the procedure. The standard formaldehyde solutions and the aqueous chromotropic acid solutions were freshly prepared on each day that calibration lines were determined. Purified chromotropic acid was used to prepare 5% aqueous solutions in deionized organic-free water. A 1.00 litre stock solution containing 4.4703 g of sodium formaldehyde bisulphite was prepared in a volumetric flask. The standard formaldehyde solutions were prepared from a solution whose formaldehyde concentration was 10 ug/mL which was prepared from the stock solution. Thus, a 1.00-mL aliquot of the stock solution was transfered to a 100-mL volumetric flask. and the volume made up to 100 mL with deionized organic-free water. Up to seven standard solutions were prepared by pipetting 0.0, 0.5, 1.0, 2.0, 3.0, 4.5 and 6.0 mL of the 10 ug/mL solution into 25-mL volumetric flasks. These were made up to 25 mL with the appropriate amounts of deionized organic-free water. These solutions correspond to

aqueous formaldehyde concentrations of 0.0, 0.2, 0.4, 0.8, 1.2, 1.8 and 2.4 ug/mL respectively.

Two-mL aliquots of each of the above seven standard solutions were pipetted into separate oven-dried clean glass 25-mL culture tubes. To each of these solutions 300 uL of aqueous 5% chromotropic acid solution was added. The solutions were thoroughly mixed by vortexing. Three mL of concentrated sulphuric acid (96%) was added to each tube cautiously in order to avoid spattering. The mixtures were thoroughly vortexed. The tubes were sealed with Parafilm and were capped loosely with Teflon-lined screw caps and were placed in a boiling water bath for 1 h. The tubes were cooled to room temperature and were again thoroughly mixed with a vortex to prevent layering prior to the removal of samples for spectrophotometric determinations. Absorbances were measured at 580 nm using 1.0 cm optical glass cells. The spectrophotometer was zeroed against analytical blanks. The analytical blanks corresponding to a 0.00 ug/mL formaldehyde solution consisted of 2.00 mL deionized organic-free water, 300 uL of aqueous 5% chromotropic acid solution, and 3.00 mL of concentrated sulphuric acid (96%). The analytical blanks were treated in an identical manner

as the solutions which contained formaldehyde. The concentration of sulphuric acid in the final solutions is 10.2 M.

b). Effect of different concentrated acids.-The exact procedures described above in a) were employed except that in order to compare the effect of different acids on the colour formation the use of perchloric acid (70%), concentrated hydrochloric acid (36%), phosphoric acid (85%), and glacial acetic acid was compared with concentrated sulphuric acid. In addition, up to six hours of heating was used so that maximum color development could be obtained. Calibration line slopes of 0.21, 0.18, 0.069, 0.00 and 0.23 AU/(ug/mL) respectively were obtained.

c). Effect of different concentrations of sulphuric acid.- The exact procedures described in a) above were employed except that in order to compare the effect of different concentrations of sulphuric acid on the colour formation the following additional procedures were conducted:

(1) 10.2 M solutions - Seven standard solutions were prepared by pipetting 0.0, 0.90, 1.8, 3.6, 5.4, 8.0 and 10.7 mL of the 10 ug/mL solution into 25-mL volumetric

flasks. These were made up to 25 ml with the appropriate amounts of deionized organic-free water. These solutions correspond to aqueous formaldehyde concentrations of 0.00, 0.36, 0.71, 1.4, 2.1, 3.2 and 4.3 ug/mL respectively. A calibration line slope of 0.21 AU/(ug/mL) was obtained after heating for 1.0 h .

(ii) 4.10 M solutions - The exact procedures described in a) above were employed except that 38% sulphuric acid obtained by diluting 4.0 mL of 96% sulphurc acid with 6.0 mL deionized organic-free water. No colour was formed after heating for 1.0 h.

(iii) Reconstituted 4.10 M solutions - Two-mL aliquots of the chromogen-containing solutions obtained in (i) which are 10.2 M in sulphuric acid were removed and each was diluted with 3.0 mL deionized organic-free water. The mixtures were thoroughly mixed by vortexing. The resulting solutions correspond to formaldehyde concentrations of 0.00, 0.14, 0.29, 0.57, 0.89, 1.3, 1.7 ug/mL respectively. No additional heating was conducted on these solutions. A calibration line slope of 0.21 AU/(ug/mL) was obtained.

(iv) Reconstituted 10.2 M solutions - To each of the solutions from (ii) which are 4.10 M in sulphuric acid, was

added 4.15 mL of concentrated (96%) sulphuric acid. The mixtures were thoroughly mixed by vortexing. The resulting solutions correspond to formaldehyde concentrations of 0.00, 0.20, 0.40, 0.80, 1.2, 1.8, 2.4 ug/mL respectively. A calibration line slope of 0.20 AU/(ug/mL) was obtained after heating for 1.0 h .

d). Oxidation-reduction experiments on sulphuric acid solutions containing developed chromogen.-To each of the reconstituted 4.10 M solutions obtained above in b) (iii) small amounts of zinc powder was added. After shaking, hydrogen gas was evolved and the purple colour gradually disappeared. The colour could be regenerated by heating these resultant colourless solutions in a boiling water bath for 1.0 h. The previously determined calibration line could be re-obtained.

e). Calibration line determination using adduct [20].-A 500 mL stock aqueous solution containing 38.6 mg of a sample of [20] was prepared in a volumetric flask. The molar mass of [20] was assumed to be 740 g/mol and the equivalent formaldehyde concentration of the stock solution was assumed to be 3.12 ug/mL. Six standard diluted solutions

containing the equivalent formaldehyde concentrations of 0.00, 0.20, 0.40, 0.80, 1.2, and 1.6 ug/mL respectively were prepared by appropriate dilutions of the stock solution with deionized organic-free water. Two-mL aliquots of each of these solutions were pipetted into separate oven-dried clean glass 25-mL culture tubes. A duplicate set of these six solutions was also prepared. To each culture tube in the first set was added 300 uL of deionized organic-free water and the solutions were thoroughly mixed by vortexing. Three mL of concentrated sulphuric acid (96%) was added to each tube cautiously in order to avoid spattering. The mixtures were thoroughly vortexed. To each culture tube of the second set was added 300 uL of aqueous 5% chromotropic acid solution and the solutions thoroughly mixed by vortexing. Three mL of concentrated sulphuric acid (96%) was added to each tube cautiously in order to avoid spattering all susequent operations were conducted as described above in (a). A control set of six standard formaldehyde solutions was prepared exactly as described in (a) except that the highest formaldehyde concentration was 1.8 ug/mL. The calibration line slopes measured were 0.096 AU/(ug/mL) for the first set of solutions prepared from

[20] which did not contain the added 5% aqueous chromotropic acid solution; 0.23 AU/(ug/mL) for the second set of solutions prepared from [20] which did contain the added 5% aqueous chromotropic acid solution; and 0.23 AU/(ug/mL) for the control set of solutions which were prepared from sodium formaldehyde bisulphite.

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